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Nutrient Content and *in Vitro* Digestibility of Apple Pomace Derived from Three Different Apple Cultivars

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ABSTRACT

This study focused on evaluating the nutritional characteristics and *in vitro* true digestibility of apple pomace derived from three apple cultivars: Golden Delicious, Starking, and Granny Smith (Malus domestica Borkh). These apple cultivars were sourced from the local market in Nigde, Turkiye. Statistical analyses, including one-way analysis of variance (ANOVA) and Duncan's test, were employed to assess variations among the apple pomace samples. Results indicated that, except for *in vitro* true digestibility, there were no significant variations in the chemical composition and total phenolic matter contents among the apple pomaces (P>0.05). However, Granny Smith apple pomace exhibited distinct features, such as higher neutral detergent fiber content (29.80%), elevated crude protein levels (5.09%) and substantial acid detergent fiber (25.30%) values. In contrast, Starking apple pomace displayed superior air-dry matter (27.24%), while Golden Delicious showcased enhanced dry matter (95.3%) and ash content (2.00%). Regarding total phenolic matter contents, Granny Smith excelled with 112.4 mg GAE/100g, outperforming Starking (103 mg GAE/100g) and Golden Delicious (75.8 mg GAE/100g). Crucially, Starking demonstrated superior *in vitro* true digestibility, with values reaching 92.36% (as received) and 92.23% (dry matter). Granny Smith, in comparison to Golden Delicious and Starking, displayed significantly different neutral detergent fiber digestibility (P<0.05). Starking apple pomace exhibit the highest overall digestibility among the apple pomaces analysed in this study, hence recommended for use in ruminant nutrition. These findings have implications for the potential utilization of apple pomace in diverse applications, given the diverse nutritional profiles of these cultivars.

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Introduction

Apple pomace, a byproduct of apple processing, is an abundant and underutilized agricultural waste material with the potential for various applications, including livestock feed, dietary fiber, and biofuel production (Eke-Ejiofor et al., 2018). The composition of apple pomace can vary significantly depending on the apple cultivar from which it is derived (Velderrain-Rodríguez et al., 2015). Determining the nutrient content and digestibility of apple pomace from different apple cultivars is of considerable interest, especially in the context of sustainable agriculture and waste reduction.

Apple pomace typically consists of apple peels, pulp and seeds, and its nutritional profile is characterized by the presence of dietary fiber, carbohydrates, antioxidants and essential minerals (Moure et al., 2001). However, the specific nutrient content can vary among apple cultivars, impacting its potential as a valuable resource in various industries, including animal nutrition and human health.

The *in vitro* digestibility of apple pomace is a critical aspect to assess, as it provides insight into its suitability as a feed ingredient for livestock, such as ruminants, non-ruminants, and monogastric animals (Sarnklong et al., 2010). The digestibility of apple pomace can influence its potential as a dietary fiber source and the extent to which its nutrients are available for absorption by the digestive systems of animals.

Previous studies have explored the nutritional profiles of apple pomace, shedding light on its potential applications in animal feed and as a source of dietary fiber (Kafilzadeh et al., 2008). These investigations have highlighted its rich content of dietary fiber, which can contribute to enhanced digestive health and improved nutritional balance in livestock diets. The apple variety itself plays a crucial role in determining the composition and potential utility of the pomace, making it essential to examine multiple cultivars to understand the full spectrum of possibilities.

While existing research provides valuable insights into the nutritional attributes of apple pomace, comparative analysis across different apple cultivars is limited. Understanding how apple variety affects the nutritional composition and *in vitro* digestibility of pomace can aid in optimizing its utilization in various sectors (Mertens, 2016).

This study focuses on investigating the nutrient content and *in vitro* digestibility of apple pomace obtained from three distinct apple cultivars. By examining the nutritional composition and digestibility of apple pomace derived from different apple varieties (S, GD and GS), this research aims to provide valuable insights into the utilization of apple pomace as a sustainable feed ingredient or dietary fiber source, contributing to both agricultural waste reduction and improved animal nutrition.

Materials and Methods

Collection and Preparation of Sample

The apple cultivars were purchased a local market in Nigde-Turkiye. An electric juice extractor was used for the sample extraction. Samples were milled (1mm sieve) and oven-dried at 50^{o}C for 48 hours before being analyzed for chemical composition and *in vitro* digestibility.

Sample Chemical Analysis

The study used established and reputable methods from AOAC (1995) to analyze the dry matter and crude ash content of apple pomaces. This choice of standardized procedures ensured the accuracy and reliability of the results, as it employed well-recognized analytical techniques for determining these components in the apple pomace samples. Crude protein in the samples was quantified using the Kjeldahl procedure, following the guidelines provided by the AOAC (1995). This involved multiplying the total nitrogen content by 6.25 to calculate CP content. The neutral detergent fiber and acid detergent fiber in apple pomaces were analyzed by Van Soest's (1991) technique. Every chemical analysis was performed in duplicate.

Total Amount of Phenolics

Using a photometric approach and the Folin-Ciocalteau reagent, the total phenolic matters content (TPC) was determined (Singleton et al. 1999). The concentration of total phenols in the apple pomace samples was measured in milligrams of gallic acid equivalents per 100 grams (mg GAE•100 g-1). This metric represents the quantity of phenolic compounds present in the samples, with gallic acid used as a reference standard. Determination of TPC involves a redox mechanism.

Determination of In Vitro Digestibilities

Samples of apple pomace from the three types of apples were subjected to digestibility testing using the DAISY^{II} incubator manufactured by ANKOM Technology (Macedon, NY), in accordance with the methodology outlined by (Goering & Van Soest, 1970). Rumen liquid from two adult Holstein cattle that were post-mortem butchered at a commercial slaughterhouse in Nigde was used to ferment AP samples, incubated at 39.5°C for 48 hours with CO_2 and buffer solutions. ANKOM^{200} fiber analyser (Ankom Technology), was used to measure NDF of the digested samples using the approach described by (Goering & Van Soest, 1970). Equations 1, 2, and 3 were used to determine the *in vitro* true DM digestibility (IVTDMD) and *in vitro* NDF digestibility (NDFD), respectively.

$$\% \text{ IVTD (as received)} = 100 - (W3-(W1\times C1)/W2\times 100 \quad (1)$$

$$\% \text{ IVTD (DM)} = 100 - (W3-(W1\times C1)/ (W2\times DM) *100 \quad (2)$$

$$\% \text{ NDFD (DM)} = 100\times[(W2\times \%\text{NDF Feed}) - (W3 - (W1 \times C1))]/ (W2 \times \%\text{DM Feed}) \quad (3)$$

Where W1 is the weight of the bag's tare, W2 is the weight of the sample and W3 is the bag's final weight after successive ND treatment and *in vitro* analysis, C1 = Blank bag correction (final oven-dried weight minus initial blank bag weight), Feed %NDF =% of NDF in Feed (%DM) and Feed %DM =% of dry matter in Feed.

Statistical Analysis

To ensure the reliability of the study, Statistical methods, including one-way ANOVA and Duncan's test, were employed to assess variations among the apple pomace samples. This analysis confirmed the normality, homogeneity and independence of the data. All statistical analyses were conducted using the software program SPSS.

Result

Nutritional Profile of Apple Pomace

Among the chemical parameters analyzed (with a significance level of $P>0.05$), there were no significant differences observed among the apple pomace samples obtained from the S, GD, and GS apple varieties (as indicated in Table 1.). However, the GS pomace sample exhibited a higher NDF value of 29.8% compared to the other two samples, which might be attributed to its thicker skin. The GD apple pomace had higher values for DM at 95.3% and ash content at 2.00% compared to the other samples. Additionally, the GS apple pomace contained more CP at 5.09% and ADF at 25.3% compared to the other apple pomaces, as shown in Table 1.

Total Amount of Phenolics Concentration

In terms of total phenolic matters concentration, there were no significant differences among the apple pomace samples ($P>0.05$). Nevertheless, the GS variety had the highest total phenolic matters content compared to the S and GD varieties, as shown in Table 2.

Digestibility Parameters

For *in vitro* true digestibility, there were significant differences between the apple pomaces at $P<0.05$. IVTD (as received) was greater in the S apple pomace at 92.36%. IVTD (on DM basis) 92.23% and NDFD 63.12% values in S apple pomace, respectively, were likewise higher. The Golden Delicious and S pomaces had considerably different ($P<0.05$) NDFD values than the GS (Table 3).

Table 1. Chemical content in apple pomace samples, expressed as a percentage

Apple Pomace	Air Dry matter	Dry matter	Ash	Crude Protein	Neutral Detergent Fiber	Acid Detergent Fiber
GD	25.19±0.019^{c}	95.30±0.031^{a}	2.00±0.172^{a}	2.57±0.074^{b}	28.00±0.309^{b}	22.70±1.020^{b}
S	27.24±0.133^{a}	95.10±0.028^{b}	1.47±0.082^{c}	2.02±0.021^{c}	20.70±0.291^{c}	15.90±0.131^{c}
GS	25.69±0.066^{b}	94.10±0.031^{c}	1.90±0.061^{b}	5.09±0.153^{a}	29.80±0.360^{a}	25.30±0.153^{a}

GD: Golden Delicious; S: Starking; GS: Granny Smith; There were no significant differences in the column (P>0.05) among the three apple pomace samples; Superscript (a, b, c) displays the sample that scored highest for each category

Table 2. The total phenolic matter content in apple pomaces

S/n	Apple Pomace	Total Phenolic Contents (mg GAE/100g)
1	GD	75.8
2	S	103.4
3	GS	112.4

GD = Golden delicious, S = Starking, GS = Granny Smith

Table 3. The parameters of apple pomaces in terms of their *in vitro* digestibility

Apple Pomace	*In vitro* true digestibility, %		
	As received	Dry matter	NDF
GD	88.17±0.789^{b}	87.47±0.782^{b}	57.11±4.13^{b}
S	92.36±0.386^{a}	92.23±0.311^{a}	63.12±1.81^{a}
GS	80.59±0.738^{c}	79.04±0.752^{c}	31.19±3.01^{c}

There were no significant differences in the column among the three apple pomace(P>0.05); GD = Golden delicious, S = Starking, GS = Granny Smith; NDF = Neutral detergent fiber; Superscript (a, b, c) displays the sample that scored highest for each category

Discussion

Nutritional Profile

In the analysis of the chemical composition of apple pomace samples from three distinct apple cultivars (Starking, Golden Delicious, and Granny Smith), it observed that the parameters assessed did not significantly differ from one another (P>0.05). However, it's worth noting that the DM content in these apple pomace samples, which ranged from 94% to 95%, was slightly higher compared to the 91.2% reported by Heuzé et al. (2020). Conversely, the CP content, ranging from 2% to 5%, was lower in this study compared to the 8% CP reported by Heuzé et al. (2020). Nevertheless, the CP values observed in this work, between 2% and 5%, were consistent with the 3.7% and 1.5% CP values reported by Albuquerque (2003) and Jin et al. (2002), respectively.

For ash content, the apple pomace samples in our study had levels ranging from 1% to 2%, which align with the 2.1% reported by Heuzé et al. (2020). The NDF values in our study varied from 20% to 29.8%, slightly lower than the 30% reported by Afzal et al. (2015) and substantially lower than the 36% NDF value reported by Preston (2014) for the same apple varieties (S, GS, and GD). In contrast, Heuzé et al. (2020) found a much higher NDF value of 65.1% for apple pomace. The ADF values in the samples, ranging from 15% to 25.3%, were within the range of 25.00% to 43.20% reported by Afzal et al. (2015) but lower than the 57.7% reported by Heuzé et al. (2020). Preston (2014) recorded an ADF value of approximately 27%, which is also within a similar range.

Overall, the findings indicate that the nutritional values of apple pomace samples from the three different apple cultivars fall within the ranges reported by previous researchers. It's important to consider that apple pomace's composition can vary due to factors like the ratio of skins, pulp, core, seeds, and juice, as well as apple variety, maturity, harvest season and extraction methods. These factors might account for the minor variations observed in the nutritional content of the apple pomace (Grigoraş, 2012; Kennedy et al., 1999).

This study aligns with previous research in terms of the nutritional composition of apple pomace and underscores the variability of this by-product, which can be influenced by various factors during apple processing. These findings are essential for assessing the potential applications of apple pomace in various industries, including livestock feed and dietary fiber production.

Total Phenolic Matter Content

In the TPC analysis, it was evident that the Granny Smith (GS) variety exhibited a higher TPC compared to the Starking (S) and Golden Delicious (GD) varieties. However, it's important to note that the TPC values in the study were lower than those reported in previous research. Specifically, the TPC values for apple pomace samples from GD, S, and GS were lower than the values reported by Er and Özcan (2010), which were 144, 143 and 132 mg GAE/100g and Vrhovsek et al. (2004), who reported values of 86.3, 131.1 and 121.0 mg GAE/100g. Similarly, the results of TPC from studies conducted by Bai et al. (2010) and Adil et al. (2007) indicated values of 62.7 mg GAE/100g and 47 mg GAE/100g, respectively, both of which were lower than what we observed in this study.

Overall, the total polyphenol content in the apple pomace samples examined in this study fell within the range reported by previous authors, with slight variations that could be attributed to their source. The diversity of polyphenols discovered in apple pomace is largely influenced by differences among various apple varieties and the conditions under which the polyphenols are extracted, including factors such as the type of medium,

temperature, pH, and duration of extraction (Cetkovic et al., 2007). Additionally, environmental conditions can significantly impact the presence of polyphenols (Jakobek et al., 2020). Various agricultural practices, such as conventional, integrated or organic farming, can also affect the polyphenol profile (Santarelli et al., 2020). Furthermore, the location of orchards can influence the color of apples and the concentration of pigments (Yuri et al., 2019).

In essence, the findings align with the broader body of research, indicating that TPC in apple pomace can vary due to factors such as apple variety, extraction methods, environmental conditions, agricultural practices, and orchard location. Understanding these variations is essential for harnessing the potential health benefits and applications of apple pomace rich in polyphenols.

Digestibility Parameters

Only limited published studies have examined the *in vitro* true dry matter digestibility (IVTD DM) of apple pomace samples derived from various apple varieties. In this study, it observed IVTD DM values ranging from 80.59% to 92.36%. These findings closely align with previous research, such as the 84-90% range reported by Kafilzadeh et al. (2008) and Anrique et al. (2002), as well as the 82-84% range documented by Singh and Narang (1992). Notably, Tagliapietra et al. (2015) reported a 98% digestibility for fresh apple pomace after hours of incubation using the gas production method, which is in a similar range to the values observed in this study.

When examining the *in vivo* organic matter digestibility of nitrogen-treated apple pomace silage in sheep, Alibes et al. (1984) found values ranging from 70% to 78%, which closely resembles the current *in vitro* results. Furthermore, in a study involving sheep fed with dried apple pomace and supplemented only with urea, the *in vivo* dry matter digestibility was 69.9%, slightly lower than the values obtained in this *in vitro* study.

These findings collectively highlight the potential of apple pomace as a valuable feed resource, particularly in terms of its digestibility. While there are variations in digestibility between *in vitro* and *in vivo* studies, our results suggest that apple pomace can serve as a promising dietary component for livestock, providing substantial nutritional value and digestibility.

Fiber Digestibility

Only a limited number of studies have delved into the NDF digestibility (NDFD) of apple pomace. In the research, it was observed that the Granny Smith (GS) variety exhibited significantly lower NDFD, as expected, given its lower dry matter digestibility ($P<0.05$). This finding is in line with a study conducted by Ahn et al. (2002), where *in vivo* testing of apple pomace revealed an NDFD of 68.4%, a value quite close to what it was observed in the Golden Delicious (GD) variety.

Mertens (2016) emphasized the presence of a robust inverse relationship between undigested NDF (i-NDF) and *in vitro* true dry matter digestibility (IVTD-DM), suggesting that i-NDF could be a preferable analytical metric, potentially replacing NDFD, to provide a more precise understanding of variations in DM degradability among constituents. Although the current study didn't explore the correlation between i-NDF and IVTD-MD, all apple pomace samples assessed in the study exhibited higher IVTD-DM as well as NDFD.

These findings shed light on the digestibility of NDF in apple pomace, with implications for its utilization as a feed resource. The identification of GS as having lower NDFD is an important observation, offering insights into the nutritional composition of different apple cultivars.

Conclusion

The GS is lower in digestibility but higher in phenolic matters and CP content. ST has the highest IVTD and NDFD digestibility. The utilization of apple pomace in ruminant nutrition offers a promising avenue for addressing various challenges in the agricultural and animal husbandry sectors. This study has provided valuable insights into the nutritional composition and digestibility of apple pomace derived from different apple cultivars. With its potential to enhance animal nutrition, reduce feed production costs and mitigate environmental concerns associated with organic waste disposal, apple pomace emerges as a viable and cost-effective feed alternative for ruminant animals.

The wide array of apple varieties and their diverse applications further underline the versatility of apple pomace as an agro-industrial by-product. Its higher phenolic matters and protein content, coupled with the varying levels of digestibility across cultivars, opens avenues for tailored feed formulations that could optimize ruminant health and performance. While certain challenges such as variations in nutritional content and the need for further research remain, the positive outcomes from this study suggest that the integration of apple pomace into ruminant diets holds great promise.

As apple processing industries continue to generate significant amounts of pomace, tapping into this resource can not only provide sustainable feed options but also contribute to the overall efficiency of the agricultural ecosystem. However, additional research is warranted to delve deeper into aspects such as optimal inclusion levels, potential interactions with other feed ingredients and the economic viability of large-scale utilization. By embracing this innovative approach to feed supplementation, ruminant nutrition can be enhanced in an environmentally responsible and economically sound manner, ultimately benefiting both the industry and the ecosystem. In summary, the use of apple pomace in ruminant feeding can offer economic advantages by reducing feed costs and providing an alternative, value-added feed source. From an environmental perspective, it contributes to waste reduction, sustainable agriculture, and potentially renewable energy production, making it a promising option for enhancing the overall sustainability of livestock operations and the food industry. In contrast to other apple pomace varieties analyzed, Starking apple pomace demonstrates the greatest overall digestibility. This is likely attributed to specific compositional characteristics, particularly its higher concentration of soluble fiber, which has the potential to enhance digestion, making it a more suitable choice for inclusion in ruminant diets.

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Abbreviations used

TPC, total phenolic composition; S, Starking; GD, Golden delicious; GS, Granny Smith; DM, dry matter; ADM, air dry matter; NDF, neutral detergent fiber; CP, crude protein; ADF, acid detergent fiber; IVTD, *in vitro* true digestibility; NDFD, neutral detergent fiber digestibility.

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Comparison of Different Soil Tillage Methods for Sustainable Agriculture in the Transition Climate Zone in Terms of Seedbed Quality and Green Grass Yield of Triticale-Vetch Mixture

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ABSTRACT

In the research conducted under the conditions of Tokat, silage triticale-vetch mixture-second crop silage corn rotation was applied. The study used four different tillage methods to compare the quality and product yield of the seedbed prepared for silage triticale-vetch mixture. Conventional tillage method (M1), conservation tillage method (M2), reduced tillage method (M3), and direct sowing (M4) methods were applied. Seedbed quality: It was evaluated regarding soil moisture content, bulk density, penetration resistance, degree of soil fragmentation, and surface roughness for depths of 0-10 cm and 10-20 cm. The effect of soil tillage methods on porosity, surface roughness, and green grass yield were statistically insignificant. Although there were statistical differences between the methods regarding soil moisture content (MC), bulk density (BD), penetration resistance (PR), and mean weight diameter values (MWD), the values are within the limit values determined for plant growth. However, crop yield is the same between soil tillage methods. This result shows that alternative tillage methods are applicable when evaluated in sustainable agriculture, which does not create a statistically significant difference in crop yield compared to conventional tillage.

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Introduction

Soil tillage is the process of regulating the condition of the soil with mechanical effects for agricultural production (Gajri et al., 2002; Özgöz et al., 2015). The creation of a favorable soil form for the cultivation of crop plants can be realized through soil cultivation (Aykas et al., 2005). The aim of tillage is to prepare a seedbed where planting can be done successfully and to effectively control weeds (Bodur, 2008). In the sowing process, the clods should be broken up more, and the soil should be pressed to make the ground where the seed is placed harder and to reduce air-filled gaps in the soil (Önal, 1995).

Qualification of the quality of a seedbed presents crucial challenges. Qualification seedbed evaluation methods are commonly classified into two categories. The first method is stated as directly measuring soil mechanical and physical properties and evaluating them in terms of yield. The second method involves combining some soil physical properties with mathematical expressions, process models, or pedotransfer functions to provide a more global assessment of the soil (Acock & Pachepsky, 1997). Soil physical properties commonly measured in the first category are penetration resistance, bulk density, particle size, mean-weight diameter, and porosity (Becher et al., 1997; Carter, 1990; Fragin, 1986; Hakansson, 1990; Luttrell, 1963; Steyn & Tolmay, 1995).

Johnson & Taylor (1960) reported that 30% of the mass of the soil in the sown layer should consist of secondary aggregates smaller than 2,5 mm in diameter. The main functions affecting plant germination and development are penetration resistance (PR), bulk density (BD), moisture content (MC), soil particle size, and surface roughness. It is reported that PR and BD values, two main criteria for soil physical quality, are widely used to decide the degree of compaction in cultivated soils and detect compacted layers (Abu-Hamdeh, 2003; Diaz-Zorita, 2000). Penetration resistance (MPa) is used to describe soil resistance. It can also provide meaningful information about the effects of soil resistance on root development and product yield. High resistance makes it difficult for plant roots to penetrate the soil and reduces root development (Barut et al., 2010).

As the soil BD increases, the moisture content, infiltration rate, and pore volume decrease, and the soil becomes less aerated. The increase in soil BD causes a reduction in plant root development (USDA-NRCS, 1996).

Compaction and particle size vary according to the soil tillage method; It affects germination, plant root development, seed contact with soil, soil aeration, and water retention capacity (Kuş & Yıldırım, 2017). As particle size values increase, soil moisture content and BD values decrease, and porosity values increase (Canbolat & Barik, 2004).

Surface roughness is a dynamic soil property that occurs on the soil surface and affects many processes (Hauer et al. 2001). Soil surface roughness is defined as irregularities in the soil surface caused by soil texture, vegetation cover, aggregate size, and land management. (Amoah et al., 2013). Tillage and meteorological conditions (wetting/drying, freeze/thaw, rainfall, etc.) change the roughness at diverse rates (Hauer et al. 2001). Soil surface roughness is affected by different tillage methods and is essential in conservation tillage methods (Bramorski et al., 2012; Vázquez et al., 2010).

Birkas et al. (2004) reported that the compaction caused by deep soil cultivation with disk tools or plows every year is at a shallow depth in the first three years, and the condensation spreads in depth from the fifth year.

Husnjak et al. (2002) compared conventional tillage, reduced conventional tillage, two different conservation tillage, and direct sowing methods in silty loam soil regarding soil physical properties and crop yield. They reported that the difference between tillage methods in terms of air and water retention capacity, porosity, and bulk density was statistically insignificant in the winter wheat period but significant in the soybean period.

Ayhan (2014) stated that soil moisture is conserved, penetration resistance and bulk density are lower, and plant emergence and product yield are better in the direct sowing application than conventional tillage method in wheat-second crop corn rotation.

Kuş & Yıldırım (2017) determined the soil moisture content, BD, soil porosity, soil resistance, change in field surface roughness ratio, MWD of the particles, and particle size distribution depending on the conditions before tillage with reduced and traditional tillage methods. They stated that minimum BD and maximum porosity values were determined with the reduced tillage method, which the tillage method affected the roughness rates at a statistically significant level, and that larger diameter particles were formed with the conventional tillage method.

This study carried out in a transitional climate zone, aimed to determine the sustainable tillage method for the region to cultivate a triticale-vetch mixture for silage. For this aim, traditional and conservation tillage methods were compared in terms of some physical properties of the soil, which indicate seedbed quality in triticale-vetch cultivation, and the effects of triticale-vetch mixture on green grass yield.

Materials and Methods

Experimental Area

The research was conducted on land belonging to the "Middle Black Sea Transitional Zone Agricultural Research Institute" in the Tokat-Kazova. Kazova is located at 40º 18′north latitude, 36º 34′ east longitude. Kazova, located between Tokat and Turhal and with an area of 29 812 ha, is a depression plain. Yeşilırmak flows through its center, and its altitude is 500-750 m above sea level (DSİ, 1974).

The research was conducted in the Yeşilırmak series, the dominant soil series in Kazova. Yeşilırmak series soils are very deep soils with a slope of 0-2%, A and C horizons, formed by the alluvium carried by Yeşilırmak. Clay content is between 36.8-42.8%. The dominant cations are Ca and Mg, and the pH is 7.72-7.90. Lime content is homogeneous along the whole profile (Oğuz, 1993).

The TAGEM project (Afacan et al., 2023) on "Comparison of Soil Properties, Yield and Energy Efficiencies in Main and Second Crop Rotations of Different Soil Processing Methods" was implemented in the trial area between 2017 and 2021. The study used experiment plots and soil tillage practices established in the TAGEM project. Silage triticale-vetch mixture was planted in the 2021-2022 production period, adhering to the applied rotation. The texture and some chemical properties of the research area soils at the beginning of the experiment are given in Tables 1 and 2.

Experiment Design and Applications

The research was carried out on 12 random plots of 50 m x 5.6 m in size, and soil tillage methods were applied in three repetitions. A 2 m space was left around the plots. The soil tillage methods detailed below were applied in the study.

M1- Conventional Tillage: Seedbed preparation was completed by using a depth of 20-25 cm with a moldboard plow, a depth of 10-15 cm with a disc harrow, and a spring tine cultivator rolling harrow combination, respectively. Sowing was done with a combined row drill.

M2- Conservation Tillage: Seedbed preparation was completed by using a depth of 20-25 cm with a chisel plow and a depth of 10-15 cm with a disc harrow, respectively. Sowing was done with a combined row drill.

M3- Reduced Tillage: Seedbed preparation was completed by processing at a depth of 15-20 cm with rotary cultivators with vertical axes. Sowing was done with a combined row drill.

M4- Direct sowing: In this method, soil tillage was not done on the parcels, and approximately fifteen days before sowing, existing weeds were killed with total herbicide (300 ml da^{-1}). Sowing was performed with a direct combined row drill.

Soil tillage and sowing practices were carried out on November 22-23, 2021. In the plots prepared by applying different soil tillage methods, 50% common vetch + 50% triticale mixture was sown at a 14 kg da^{-1} sowing rate and planted at a 3-4 cm depth. DAP at 20 kg da^{-1} norm was applied to all parcels as base fertilizer upon planting. Ammonium Sulphate (21% nitrogen and 24% sulfur) at 18 kg da^{-1} seeding rate was used as the top fertilizer on April 05, 2022. Harvesting was done on May 30, 2022.

Sampling and Measuring Soil Properties

To determine the quality of the seedbed, bulk density, penetration resistance, soil moisture content, degree of soil fragmentation, and surface roughness were measured after sowing. All samples and measurements were carried out in three replications in each plot. No sampling was done from wheel tracks.

Table 1. Textural characteristics of study area soils*

Soil Tillage Method	Depth (cm)	Texture (%)			Texture Class
		Sand	Clay	Silt	
M1	0-10	26.76	35.76	37.48	Clay loam
	10-20	27.15	34.65	38.20	Clay loam
M2	0-10	26.27	35.55	38.18	Clay loam
	10-20	26.52	35.08	38.40	Clay loam
M3	0-10	26.06	35.31	38.63	Clay loam
	10-20	26.68	34.65	38.67	Clay loam
M4	0-10	26.26	36.24	37.50	Clay loam
	10-20	26.94	33.95	39.11	Clay loam

Notes: M1, Conventional tillage; M2, Conservation tillage; M3, Reduced tillage; M4, Direct sowing. *Afacan et al. (2023)

Table 2. Some chemical properties of the study area soils at the beginning of the experiment*

Soil Tillage Method	Depth (cm)	Electrical Conductivity (mmhos /cm)	Salt (%)	pH	Lime (%)	Available Phosphorus (kg da^{-1})	Available Potassium (kg da^{-1})	Organic Matter (%)
M1	0-10	0.86	0.03	7.82	10.59	4.91	62.87	2.31
	10-20	0.81	0.03	7.80	10.98	2.54	65.66	2.37
M2	0-10	0.90	0.04	7.83	10.98	4.28	75.44	2.28
	10-20	0.85	0.03	7.82	10.59	2.18	67.79	2.19
M3	0-10	0.94	0.04	7.82	10.33	4.15	77.17	2.62
	10-20	0.85	0.03	7.81	11.50	2.52	67.49	2.23
M4	0-10	0.96	0.04	7.74	10.98	5.52	82.14	2.82
	10-20	0.91	0.04	7.71	10.72	4.52	65.59	2.48

Notes: M1, Conventional tillage; M2, Conservation tillage; M3, Reduced tillage; M4, Direct sowing.

Seventy-two undisturbed soil samples (12 plots × 3 repetitions × 2 depths) were taken from two depths (0-10 cm and 10-20 cm depths) using cylinders of 0.05 m diameter and 0.05 m height to determine soil bulk density and moisture content. The samples brought to the laboratory were weighed, left to dry in an oven at 105ºC for twenty-four hours, and weighed again (Vepraskas & Wagger, 1989). Moisture content and BD were determined using dry and wet weights of soil samples (Baver et al., 1972; Demiralay, 1993).

To determine the soil penetration resistance of soils, measurements were made using a digital penetrometer, which can measure up to 45 cm depth at 2.5 cm intervals. A conical tip with a diameter of 12.7 mm was used in the measurements. At each measurement point, the average penetration resistance values were obtained for 0-10 cm depth by averaging the values measured at 0, 2.5, 5, 7,.5, and 10 cm depths and for 10-20 cm depth by averaging the values measured at 12.5, 15, 17.5 and 20 cm depths. Porosity values of soil samples were calculated using Equation 1 (Erbach, 1987).

$$P = 1 - (BD/ P_k) \quad (1)$$

Where P is porosity, BD is bulk density (g cm^{-3}), and P_k is soil particle density (2.65 g cm^{-3}).

Approximately 5 kg of soil samples were taken from each plot in 3 replicates with the help of a shovel from the soil particle size measurement depths of the created seedbed (0–10 cm and 10–20 cm) and were transported to the laboratory without being disturbed. These air-dry samples were kept in the laboratory for two months, and sieve analysis was performed (Altıkat, 2005; Çelik, 1998). Sieve analysis used sieves with 63, 32, 16, 8, 4, 2, and 1 mm hole diameters specified in Anonymous (1974). First, the sieving time and frequency required to prevent the soil from crumbling and ensure full-size distribution were determined. Accordingly, the sieving process was carried out by applying a 30 s elimination time and 50 Hz vibration frequency (Anonymous, 1980). To express the soil fragmentation fractions after tillage and to evaluate the seedbed quality, the soil particle sizes obtained were based on three groups:>8 mm, 1-8 mm, and <1 mm (Çelik, 1998). Moreover, the mean weight diameter values were also determined using Equation 2 (Adam & Erbach, 1992; Demir et al., 1996).

$$MWD = \Sigma X_i.W_i \quad (2)$$

Where W_i is the sample weight of each size fraction (g), X_i is the mean diameter of each size fraction (mm), and MWD is the mean-weight diameter (mm).

A profilometer consisting of rods placed on a 1 m long profile at 2.5 cm intervals was used to determine the roughness of the soil surface created by soil tillage methods. The profilometer was placed perpendicular to the direction of the sowing, and measurements were made in three replications in each plot. Surface roughness was determined using Equation 3 (Çarman, 1997).

$$R = 100.Log10\ S \quad (3)$$

Where S is the standard deviation, and R is surface roughness (%).

Crop Yield

In the study, the effect of tillage methods on green grass (silage) yield of the triticale-vetch mixture was also determined in addition to seedbed quality. To determine the green grass yield of the triticale-vetch mix, the plants harvested in a 1 m^2 area in three replicates in each parcel were weighed, and the green grass yield per decare (kg da^{-1}) was determined.

Statistical Analysis

First, the Kolmogorov-Smirnov (KS) test was performed on the data set created for each parameter to determine whether the data set showed a normal distribution. Then, normally distributed data sets were subjected to analysis of variance and Duncan's multiple range test. Statistical analyses were performed using the SPSS 17.0 (SPSS, 2017). As a result of these statistical analyses, the most appropriate tillage method was selected by determining the effect of tillage methods on crop yield and some soil properties used to express the quality of the seedbed.

Results and Discussion

Effects of Tillage Methods on Soil Physical Properties

Tillage methods significantly affected the MC values determined at a 0-10 cm depth at the P<0.05 level, with no significant effect at 10-20 cm. According to soil tillage methods, moisture content values varied between 22.95% (M3) - 29.03% (M4) and 22.38% (M3) - 27.46% (M4) at depths of 0-10 cm and 10-20 cm, respectively. It was observed that the main difference in moisture content values at the surface depth, where soil tillage methods had a statistically significant effect, was due to M4, and the other three tillage methods were statistically in the same group (Table 3).

Tillage methods affected the bulk density values measured at two depths (0-10 cm and 10-20 cm) at P<0.01. Soil tillage according to the mean bulk density values, tillage methods were ranked as M4>M1>M3>M2 at depths of 0-10 cm and M4>M2>M1=M3 10-20 cm at depths (Tables 3 and 4).

Hakansson & Lipiec (2000) stated that plant growth is limited when the BD is 1.60 g cm^{-3}, and the limiting BD value varies according to soil texture. Lhotsky et al. (1984) stated that the limit BD value for clay loam soils is 1.40 g cm^{-3} (Badalikova, 2010). Additionally, Pierce et al. (1983) stated that in clay loam soils, the ideal BD is <1.40 g cm^{-3}, the BD values that negatively affect the development of roots are 1.63 g cm^{-3}, and the BD that prevents the growth of roots is >1.80 g cm^{-3}. According to these values, it was determined that the BD values measured in the research area, which has a clayey loam texture, were generally below the values that limit plant root development and were close to the limit values at both depths, especially in the M4 treatment (Tables 3 and 4).

Barut et al. (2010) reported that BD increased in all methods at a depth of 0-10 cm, while it increased only in the direct sowing method at lower depths; Gürsoy & Kolay (2012) reported that the BD value was higher in conventional tillage method; Kuş & Yıldırım (2017) stated that the lowest BD values were determined in the reduced tillage method. So et al. (2009) reported that zero tillage did not change the BD in the short term but decreased the BD in the long term. Bulut (2018) reported that the highest and lowest humidity values were in the direct sowing and conventional tillage methods, respectively. In the study, similar to the results reported in the literature, it was determined that soil MC and BD were higher in the M4.

According to the results of variance analysis, the effect of tillage methods on soil porosity values at both depths is statistically insignificant (Tables 3 and 4). Maximum porosity at the surface depth was obtained in the M2 treatment (53.92%) and minimum porosity in the M4 treatment (47.28%). Bahtiyar (1996) states that the porosity value of soil in texel structure varies between 24.5% and 47.5%. Porosity and BD are parameters that can change with tillage, and their relationship is opposite. An increase in one means a decrease in the other (Ülger et al., 2002). In this study, when BD and porosity values are analyzed together, it is seen that they have an opposite relationship. M3 and M2 caused looser soil structure and higher porosity than M1 and M4.

According to the results of variance analysis, it was determined that tillage methods significantly affected soil PR values at the level of P<0.05 at the surface depth and at the level of P<0.01 at the depth of 10-20 cm. When the average soil PR values were analyzed, it was determined that the highest value was obtained in the M4 (1.2 MPa). The smallest value was obtained in the M3 (0.4 MPa) method at 0-10 cm depth, and M1, M2, and M3 methods were statistically in the same group. At the second measurement depth (10-20 cm), the maximum value was obtained in the M2 (1.7 MPa) and the minimum value in the M1=M3 (0.8 MPa) methods. At this depth, M1-M3 and M2-M4 methods were statistically in the same group (Table 4).

Table 3. Some physical properties of study area soils under different tillage methods (0-10 cm)

Soil tillage method	Moisture Content (%)	Bulk density (g cm^{-3})	Porosity (%)	Penetration resistance (MPa)
M1	23.18±2.55b	1.30±0.10b	48.86±3.56	0.6±0.20b
M2	23.28±1.61b	1.19±0.07d	53.92±1.06	0.6±0.15b
M3	22.95±1.27b	1.25±0.15bc	50.84±5.58	0.4±0.29b
M4	29.03±9.79a	1.45±0.09a	47.28±6.65	1.2±0.17a
F value	2.95*	9.60**	1.10^{ns}	6.95*

Notes: *, significant at the 0.05 level; **, significant at the 0.01 level; ^{ns}, not significant. There isn't a statistical difference between the values shown with the same letter in the columns. M1, Conventional tillage; M2, Conservation tillage; M3, Reduced tillage; M4, Direct sowing.

Table 4. Some physical properties of study area soils under different tillage methods (10-20 cm)

Soil tillage method	Moisture Content (%)	Bulk density (g cm^{-3})	Porosity (%)	Penetration resistance (MPa)
M1	23.07±2.02	1.33±0.09b	51.16±2.63	0.8±0.13b
M2	22.41±1.38	1.44±0.06a	45.63±1.56	1.7±0.18a
M3	22.38±0.81	1.33±0.12b	47.10±2.26	0.8±0.43b
M4	27.46±8.55	1.51±0.10a	46.22±2.06	1.6±0.23a
F value	2.69^{ns}	7.72**	3.98^{ns}	10.10**

Notes: **, significant at the 0.01 level; ^{ns}, not significant. There isn't a statistical difference between the values shown with the same letter in the columns. M1, Conventional tillage; M2, Conservation tillage; M3, Reduced tillage; M4, Direct sowing.

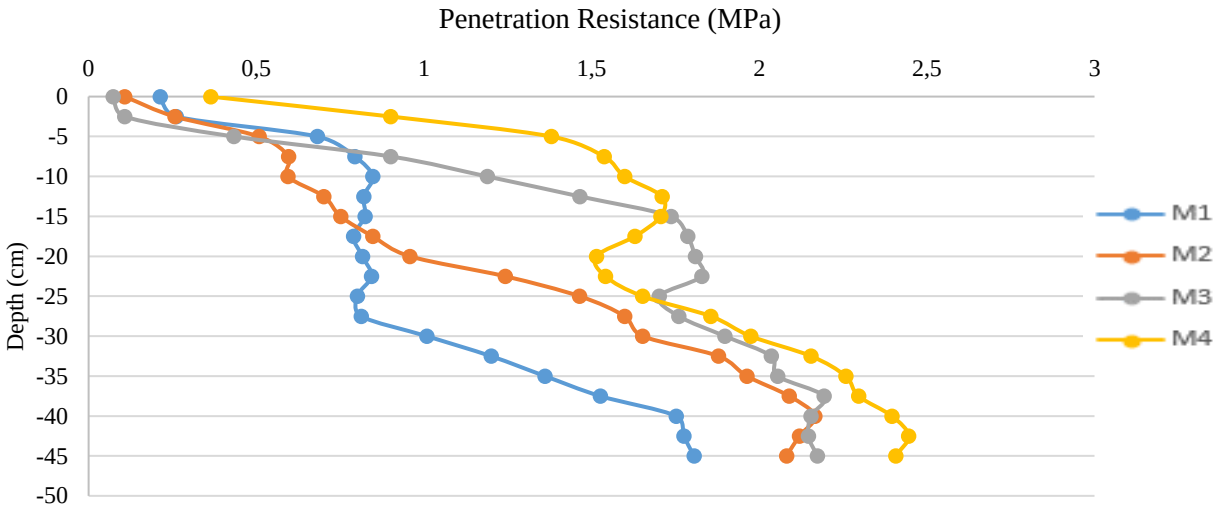

Figure 1. Variation of penetration resistance with depth (M1, Conventional tillage; M2, Conservation tillage; M3, Reduced tillage; M4, Direct sowing)

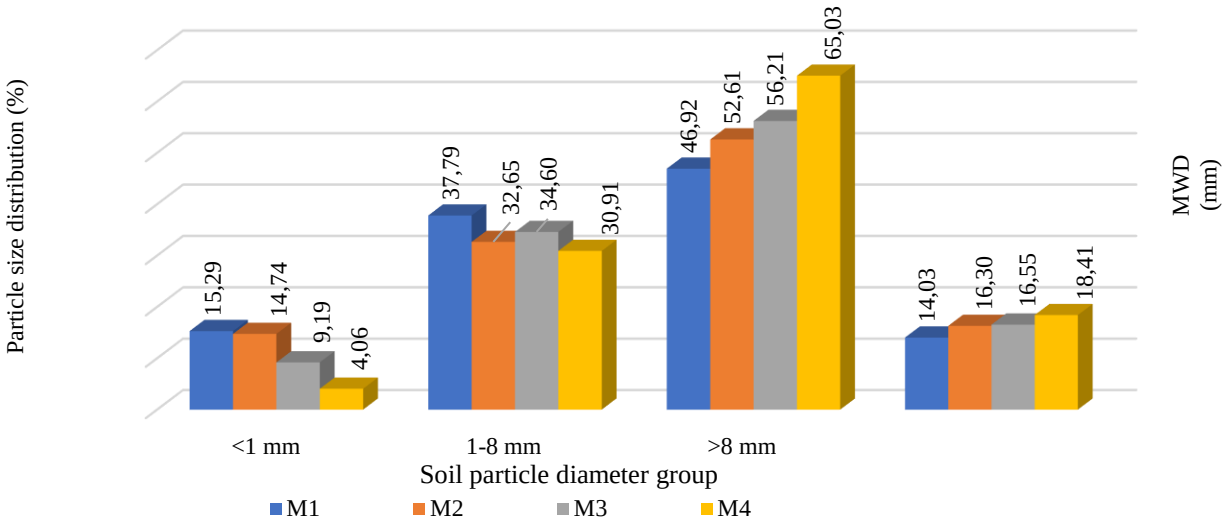

Figure 2. Soil particle size distribution (%) for 0-10 cm depth after tillage (M1, Conventional tillage; M2, Conservation tillage; M3, Reduced tillage; M4, Direct Sowing; MWD, Mean weight diameter)

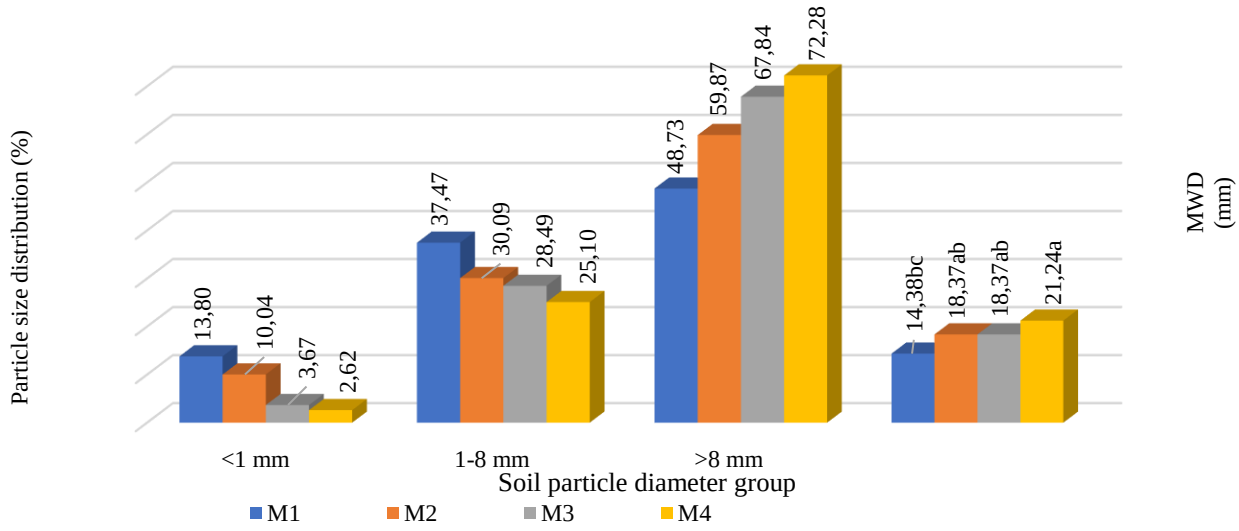

Figure 3. Soil particle size distribution (%) for 10-20 cm depth after tillage (M1: Conventional tillage, M2: Conservation tillage, M3: Reduced tillage, M4: Direct Sowing, MWD: Mean weight diameter)

The variation of soil PR with depth is given in Figure 1. When the change in PR with depth is examined, it is generally seen that the PR is lower in the M1 and higher in the M4 plots. In addition, there are no compacted layers (soil pan) in the study area, which would negatively affect plant production.

Hakansson & Lipiec (2000) stated that the limiting value of PR, which prevents root development in plants, is 3 MPa; Ehlers et al. (1983) noted that this value will vary according to the tillage method and can be used as 3.6 MPa and 5 MPa in soils where conventional tillage and direct seeding are applied, respectively. Bengough et al. (2005) accept the critical value as 2 MPa in soils without continuous root channels and cracks. However, Sa et al. (2014) reported that the limit value of 2 MPa cannot be accepted as the value limiting root development for different tillage methods and that 2 MPa should be used when the direct sowing method is applied in soils with high clay content, 3 MPa when chisel tillage is applied, and 3.5 MPa when M4 is applied (Çelik et al., 2019). The soil PR values determined in the research are generally lower than the accepted limit values for plant root development.

Gürsoy & Kolay (2012) measured the penetration resistance of the soil at 0-10 cm, 10-20 cm, and 20-30 cm depths. Researchers determined it was higher in the direct sowing method for the first two depths and in the conventional tillage method for the final depth. In the direct sowing method, Küçükalbay & Akbolat (2015) determined the maximum PR value at 0-20 cm soil depth.

The optimum seedbed is the presence of finer and pressed soil around and below the seed and coarser and unsubmerged soil above the seed (Keçecioğlu & Gülsoylu, 2002). For proper agricultural technique and successful planting, it is desired to have more soil particles with an average diameter of around 10 mm in the prepared seed bed (Gökçebay, 1986). To make a generalizing evaluation in the study, three groups, >8 mm, 1-8 mm, and <1 mm, were taken as the basis (Çelik, 1998) (Figure 2 and Figure 3). Accordingly, it was observed that particles larger than 8 mm were more and particles smaller than 1 mm were less in all soil tillage methods at depths of 0-10 cm and 10-20 cm. According to the proportion of particles<1 mm, 1-8 mm, and >8 mm at 0-10 cm depth, tillage methods were M1>M2>M3>M4, M1>M3>M2>M4 and M4>M3>M2>M1, while M1>M2>M3>M4, M1>M2>M3>M4, and M4>M3>M2>M1 for 10-20 cm depth.

While there was no statistically significant difference between the treatments at 0-10 cm depth, the difference between the treatments at 10-20 cm was statistically significant at $P<0.05$ level. MWD values ranged between 14.03 - 18.41 mm at 0-10 cm depth and between 14.38 - 21.24 mm at 10-20 cm depth. As expected, the MWD values were more significant in the plots where the M4 was applied.

To ensure proper water movement in the soil and avoid soil erosion (Hu et al., 2011), at least 50% of the soil grain size distribution must consist of particles in the range of 3.17-6.35 mm (Baver et al., 1972). Soil fragments vary depending on soil moisture content, texture, organic matter content, and the type and characteristics of the tillage tool.

The study reported that the particles suitable for plant growth were higher in the M1 method than in other methods. It is seen that this result also affects the mean-weight diameter of the soil. It was determined that the MWD values were higher in the M4 method. MWD value was found to be higher than other methods due to the higher proportion of aggregates with diameters larger than 8 mm. Kuş & Yıldırım (2017) stated that the aggregate ratio with a diameter less than 8 mm was higher in the reduced tillage method.

Effects of Tillage Methods on Surface Roughness

One of the essential parameters in seedbed preparation is surface roughness. In the analysis of variance to determine the effect of soil tillage methods on surface roughness values, the effect of tillage methods on the surface roughness of the soil was found to be statistically insignificant (Table 5).

The highest surface roughness was determined in the M4 treatment, and the lowest surface roughness was determined in the M2 treatment. When examined in terms of functional and structural properties, it was determined that the M4 treatment had the highest average values (26.44%). Kuş & Yıldırım (2017) reported that the surface roughness values determined from M1 and M3 were statistically different, and roughness rates obtained in conventional tillage were higher.

Effects of Tillage Methods on Green Grass Yield

The analysis of variance test conducted to determine the effect of tillage methods on green grass yield of silage triticale-vetch mixture showed that the effect of tillage methods on the yield was statistically insignificant (Table 6). Tillage methods are ranked as M4>M1>M2>M3 regarding green grass yield of triticale-vetch mixture for silage. Similarly, Yalçın et al. (1997) and Zeren et al. (1993) reported that the yield increased in the direct sowing method compared to other methods. Stipešević et al. (2019) found that the yield values of Sudan grass were higher in the first year in the conventional tillage method and in the second year in the reduced tillage method in which they used a disc harrow.

Table 5. Surface roughness values of study area soils under different tillage methods (%)

Soil tillage method	Surface roughness (%)
M1	24.32±7.96
M2	15.45±7.03
M3	20.08±12.23
M4	26.44±11.12
F value	1.98^{ns}

Notes: ^{ns}, not significant. M1, Conventional tillage; M2, Conservation tillage; M3, Reduced tillage; M4, Direct sowing.

Table 6. Green grass yield values under different tillage methods (kg da^{-1})

Soil tillage method	Green grass yield (kg da^{-1})
M1	2280±428.63
M2	2234±344.52
M3	2156±187.76
M4	2748±692.83
F value	2.67^{ns}

Notes: ^{ns}, not significant. M1, Conventional tillage; M2, Conservation tillage; M3, Reduced tillage; M4, Direct sowing.

Conclusion

This study evaluated soil physical properties and product yield to determine the sustainable tillage method for the region to cultivate triticale-vetch mixture for silage. Soil moisture content (MC) is critical for plant growth, and excess or insufficient water can negatively affect plant growth. Furthermore, optimum moisture content (MC) optimizes the interaction of tillage machines with the soil and prevents soil compaction. Penetration resistance (PR) indicates the effectiveness of tillage and how easily plant roots can move through the soil. Surface roughness and soil particle size are also important parameters for seedbed quality.

It was determined that the conventional tillage method was the most sustainable seedbed for plant growth. However, the effect of soil tillage methods on porosity, surface roughness, and green grass yield were statistically insignificant. The highest green grass yield was determined in the direct sowing method. Although there were statistical differences between the methods regarding bulk density, moisture content, penetration resistance, and mean weight diameter values, the values are within the limit values determined for plant growth. Although conventional tillage and seedbed preparation is one of the essential steps in plant growing processes, alternative methods have been developed in recent years within the scope of sustainable agricultural practices to protect natural resources and maintain soil health. The study results showed that the conservation tillage method is preferable to the conventional tillage method. The results obtained are essential for future generations to gain maximum benefit from agricultural soils, ensure sustainable agriculture, and minimize adverse effects on the environment. To determine sustainable soil tillage methods that can be adapted to climate change, studies should be carried out in which soil properties, crop properties, energy efficiency, management, and economic aspects of the methods are considered.

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Determination of Yield and Quality Characteristics of Some Fodder Beet (*Beta vulgaris* L. var. *rapa*) Varieties in Sakarya Ecological Conditions

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ABSTRACT

This research; it was established under the Sakarya ecological conditions and carried out for 2 years between 2021 and 2023 to determine the yield and quality characteristics of some fodder beet varieties. The experiment was set up with four replications in a randomized blocks trial design. Rekord, Rota, Ursus and Zentaur varieties were used in this research. In the study; in tubers; length (cm), diameter (cm), aboveground length ratio (%), yield (kg/da), dry matter content (%), dry matter yield (kg/da), crude protein ratio (%), sugar ratio (%) and weight loss in storage (%) and in leaves; yield (kg/da), length (cm), width (cm), dry matter ratio (%), dry matter yield (kg/da) and crude protein ratio (%) properties were investigated. The most positive data in the study were obtained from the Ursus variety (In tuber: length; 29.1 cm, yield; 19.309 kg/da, dry matter content; 15.9%, crude protein ratio; 9.30%, sugar ratio; 6.35%, and in leaves; length; 61.0 cm, yield; 2.585 kg/da, dry matter content; 14.0%, crude protein ratio; 23.5%).

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Introduction

The nutrition of our Turkiye's animals is largely based on pasture. However, the productivity of the pastures is quite low due to excessive and uncontrolled grazing. For this reason, it is necessary to increase the production amounts of forage crops in field agriculture in order to reduce and improve the grazing pressure on pastures and to meet the required quality of forage.

Turkiye has a total area of 19.8 million hectares of field agriculture, of which 1.8 million hectares are cultivated with forage crops. Although the ratio of forage crops in field agriculture has increased in recent years, it is around 9%, and agriculture and animal husbandry are quite low compared to developed countries (Celik, 2013).

The annual roughage requirement of the Turkiye's livestock is 55 million tons. 10 million tons of this amount are tried to be met from pastures, 35 million tons from forage crop agriculture and the remaining 10 million tons from factory-produced mixed feeds (Celik, 2013). The main issue here is not the quantity but the quality of the feed obtained from pastures and forage crops. When the situation is evaluated from this perspective, it is obvious that there is a deficit in quality roughage. Among the forage crops, the cultivation area of fodder beet is approximately 11.15 hectares (Anonymous, 2024). It is an important forage plant that can provide the highest yield per unit area and is rich in water. It is an important forage plant, especially for dairy farming; it increases the quality of milk, the fat and crude protein ratio, and saves concentrated feed. Because it is delicious, it is easily consumed by animals. Its digestibility level is high (87-93%). The rate of nutrients in dry matter is high. It provides more energy than other forage plants. Its leaves are also used in animal feeding and strengthen the digestive systems of animals (Acikgoz, 2021; Akyildiz, 1986; Anonymous, 2024; Ergul, 1988; Genckan, 1983).

Many researchers (Abou-Deya, 1991; Acar, 2000; Adiyaman, 2003; Albayrak & Yuksel, 2009; Anonymous, 2002; Avcioglu & Sabanci, 1993; Bartolomaeus, 1988;

Cristiansen-Weniger et. al., 1979; Cetin & Ozhan, 1992; Cetin, 1998; Elmali, 1998; Geren, 1996; Hofman et. al., 1970; Jankowiak et. al., 1988; Kampf et. al., 1985; Kokten & Ozdemir, 2020; Manga, et. al., 1997; Oz, 1997; Ozdemir & Kokten, 2020; Salisbury & Ross, 1992; Sedlmayr, 1966; Senf, 1961; Soya et. al., 1997; Voighlander & Jacob, 1987; Yilmaz, 2018;) working with fodder beet have given the following information on the subject: tuber yield is 5-20 tons/da, leaf yield is 1-4 tons/da, dry matter ratio is 8-28%, sugar content is 3-8%, and crude protein ratio is 5-10%.

The aim of this research is to determine the yield and yield factors of forage beet, which yields very high yields per unit area, in order to close the quality forage deficit in animals.

Materials and Methods

Climate Characteristics of the Research Area

The research was conducted in the Karapınar neighborhood of Adapazarı district (40° 47' 20¨ N, 30° 24' 21¨ E, and altitude 31 m), Sakarya province, located in the Eastern Marmara region. Climate data were taken from the Adapazarı Meteorology Station, which is approximately 14 km away from the research area. The periods when the research was conducted and the climate data for the long-term average (L.T.A.*) are given in Table 1.

In the first year when the experiment was conducted, the total rainfall (April-September) was 388 mm, the average temperature was 20.1 °C, and the relative humidity rate was 73.8%, while in the second year for the same period, these values were 248.5 mm, 20.3 °C, and 71.4%, respectively. The long-term average is 235.4 mm, 20.4 °C, and 68.7%, in the same order. In this case, the rainfall and relative humidity of both years in which the experiment was conducted are higher than the long-term average, while the average temperatures are very close to the long-term average.

Soil Properties of the Research Area

Soil samples taken from 0-20 and 20-40 cm depths of the research area were analyzed in the laboratory of Pamukova Vocational School and are given in Table 2.

The analysis results showed that the soil of the trial area had a clay loam structure at depths of 0-20 and 20-40 cm, showed a slightly acidic reaction in terms of pH value, did not cause any problems in terms of salinity, and was poor in terms of lime.

The soils of the research area, which are moderate in terms of nitrogen, are insufficient in terms of organic matter, useful phosphorus, and potassium (Brohi & Aydeniz, 1991). At depths of 20-40 cm, it is generally observed that nutritional elements gradually decrease.

Establishment and Evaluation of the Research

Establishment of the trial and parceling: The trial was established on April 10, 2021 in the first year and on April 10, 2022 in the second year. The plots were prepared and planted with 50 cm spacing between rows, 30 cm spacing between rows (Acikgoz, 2021), 16 plants per row of 5 m length, and 6.670 plants per decare.

Seed material: In the research, Rekord, Rota, Ursus, and Zentaur fodder beet varieties were used.

Cultural procedures: When the plants completed their germination and had 2-3 leaves, the misting process was carried out. In the trial; 15 kg/da triple super phosphate (TSP) and 30 kg/da ammonium nitrate (AN) fertilizer were applied. The entire TSP and 10 kg of AN were given together with the planting, and the other 20 kg of AN was given as 10 kg for both hoeing operations (Yilmaz, 2018). The experiment was hoeed a total of 3 times and watered as necessary.

Harvest and storage: In both years, the harvest was made on October 15, when root growth stopped, the leaves dried and drooped, and the middle leaves began to turn yellow (Acikgoz, 2021). The vegetation period between planting and harvest is 185 days. Plant samples were taken from 20 plants in the middle, excluding 3 plants from each side of the 4 rows in the middle (2 rows in each plot); 10 of them were used for measurement and weighing, and the other 10 were stored in an unheated indoor area to determine storage losses. The storage period is 175 days, from harvest on October 15 to April 10, when the animals begin to access fresh green fodder in the spring. The tubers, which were weighed and stored on October 15, were weighed again on April 10, and the fresh weight losses of the tubers were determined by proportioning the differences to the first weighing figures.

Features examined: In the research; length (cm), diameter (cm), above-ground length ratio (%), yield (kg/da), dry matter content (%), dry matter yield (kg/da), crude protein ratio (%), sugar ratio (%) and weight loss in storage (%) were studied in the tuber. Additionally, in the leaf (6 features), length (cm), width (cm), yield (kg/da), dry matter content (%), dry matter yield (kg/da) and protein rate (%) were studied. Among the quality analyses, the crude protein ratio was made according to the Kjeldahl method and the sugar ratio was made according to the Betalyser method (Akyildiz, 1986; 2004).

Evaluation of the data: The experiment was set up with four replications according to the Randomized Blocks Trial Design, the statistical analysis of the obtained data was made in the TOTEMSTAT statistical program (Acikgoz et. al., 2004) and the Least Significant Difference (LSD, 5%) values are given below the tables.

Table 1. The climate dates of the trial area

Years	Total Precipitation (mm)	Average Temperature (ºC)	Relative Humidity (%)
2021-22	388.0	20.1	73.8
2022-23	248.5	20.3	71.4
L.T.A.*	235.4	20.4	68.7

(*): Meteorological Station Adapazarı/Sakarya.

Table 2. Soil properties of of the trial area

Properties	Sample Depth (cm)	
	0-20	20-40
Structure	loamy	loamy
pH	6.61	6.91
Total salt (%)	0.024	0.023
$CaCO_3$ (%)	5.61	7.58
Organic matter (%)	0.91	0.94
Nitrogen (kg ha^{-1})	0.58	0.61
P_2O_5 (kg ha^{-1})	8.5	7.5
K_2O (kg ha^{-1})	195.0	225.0

Results

Tuber Properties

Tuber length (cm)

Tuber length values, which are one of the clear indicators of high yield, are given in Table 3. The longest tuber length was taken from the Ursus variety in both years and on average, and the shortest was from the Zentaur variety.

The number of 6.670 plants per square meter represents close to ideal plant density, and therefore the tubers were able to show their real performance in terms of length.

Tuber length data are close to the values of Oz (1997), Acar (2000) and Yilmaz (2018) and higher than the data of Abou-Deya (1991), Geren (1996) and Adiyaman (2003).

Tuber diameter (cm)

The data obtained from the measurements are presented in Table 3. According to the variety average, the widest diameter was measured in the Ursus variety at 14.6 cm, and the narrowest diameter was measured in the Zentaur variety at 11.8 cm.

In terms of years, the first year data (13.5 cm) is higher than the second year (12.6 cm). In terms of variety × year interactions, the Ursus variety gave the highest number with a diameter of 15.1 cm in the first year of the study.

Tuber diameter, which is a quantitative character and directly proportional to tuber length, is one of the most important components that make up tuber yield, and as plant density increases, tuber diameter decreases. The most suitable tuber diameter for machine harvesting is stated as 8 cm, and a tuber diameter that is too large is not desired.

The data obtained are similar to the data of Abou-Deya (1991), Acar (2000), Elmali (1998), and Yilmaz (2018), and are higher than the data of Adiyaman (2003), Geren (1996), and Oz (1997).

Tuber aboveground length ratio (%)

The datas obtained are given in Table 3. The highest rate was determined in the Ursus variety with 64.4% and the lowest in the Zentaur variety with 52.6%. It was determined that the first year data was higher than the second year data in the values between years.

It is expected that the above-ground growth rate of the tuber will be similar to the tuber length values. It is known that fodder beet has high above-ground growth rates; therefore, it has been reported by Senf (1961) that their resistance to drought and cold is less Kampf et. al., (1985) and that large beets with external roots are more suitable for clayey soils.

The data obtained are close to the data of Adiyaman (2003), Oz (1997), and Yilmaz (2018), and higher than the data of Abou-Deya (1991), Anonymous (2002), and Geren (1996).

Tuber yield (kg/da)

Tuber yield values, which are the most important yield parameter, are given in Table 3. According to the variety averages, the highest yield was obtained from the Ursus variety with 19.309 kg/da, and the lowest yield was from the Zentaur variety with 12.845 kg/da. The first year data of the research is higher than the second year. In terms of variety × year interactions, it is seen that the Ursus variety gave the highest yield (19.958 kg/da) in the first year.

The weights of the tubers from which data were taken in the research were between 1.085 g (7.237 kg/da), and 4375 g (29.181 kg/da) and the average weight was determined as 2.730 g (18.209 kg/da). Tuber yield depends on the genetic capacity of the variety and the suitability of climate and soil conditions.

The datas obtained are in line with the findings of Acikgoz (2021), Avcioglu & Sabanci (1993) and Soya et al., (1997), close to the findings of Cetin (1998), Oz (1997), and Yilmaz (2018), while it is higher than the data of Abou-Deya (1991), Adiyaman (2003), Anonymous (2002), Bartolomaeus (1988), Cetin & Ozhan 1992), Elmali (1998), Geren (1996), Manga et al., (1997) and, Ozdemir & Kokten (2020).

Tuber dry matter content (%)

The results obtained from the weighing and proportioning of the dried samples are given in Table 3. The Ursus variety gave the highest dry matter content in both years and on average. In terms of years, first year data was higher than second year data.

Dry matter content; it is very important economically in animal feeding, silage making, and fresh storage (Akyildiz, 1986; Ergul, 1988; Geren, 1996; Soya et. al., 1997). Climate data and plant density during the year have a great impact on dry matter formation, as well as the genetic structure of the varieties. While tuber yield increases in rainy years and irrigated conditions, dry matter content decreases (Jankowak et. al., 1988).

The data obtained in the research (total precipitation and dry matter content in the first year: 988.7 mm, 13.8%, and in the second year, 781.4 mm and 14.7%) confirm this thesis. Some researchers (Akyildiz, 1986; Hofman et. al., 1970) report that there is a negative relationship between dry matter ratio in tubers and tuber yield.

The data obtained are close to the results of many studies (Abou-Deya, 1991; Anonymous, 2002; Bartolomeus, 1988; Ergul, 1988; Geren, 1996; Voighlander & Jacob, 1987; Yilmaz, 2018) and higher than the results of some studies (Adiyaman, 2003; Cetin, 1998).

Tuber dry matter yield (kg/da)

The data obtained by multiplying herbage yield and dry matter content are given in Table 3. The highest dry matter yield was obtained from the Ursus variety, both in terms of variety and variety × year interactions. Depending on the herbage yield, the first year data is higher than the second year data.

The research findings are consistent with Geren (1996) and Yilmaz (2018), lower than Bartholomaeus (1988), and higher than Adiyaman (2003) and, Ozdemir & Kokten (2020).

Tuber crude protein ratio (%)

The determined crude protein ratios are given in Table 3. The highest values were taken from the Ursus variety in both years and on average (9.02, 9.58, and 9.30%). First year data (8.41%) is higher than second year data (8.79%).

The data obtained are consistent with the findings of Acar (2000) and Adiyaman (2003), and higher than the findings of Cetin (1998) and Ergul (1988) and, lower than Ozdemir & Kokten (2020).

Tuber sugar ratio (%)

The obtained figures are given in Table 3. The highest sugar content was obtained from the Ursus variety in both years and on average, and the lowest was from the Zentaur variety. Second year data (5.69%) was higher than first year data (5.38%).

Table 3. Data on tuber properties obtained in the study

Plant Varieties	Tuber Properties								
	Length (cm)			Diameter (cm)			Aboveground length (%)		
	1. Year	2. Year	Mean	1. Year	2. Year	Mean	1. Year	2. Year	Mean
Rekord	26.9	25.7	26.3	13.3	12.1	12.7	56.5	54.4	55.5
Rota	28.9	27.8	28.4	13.2	12.9	13.1	58.7	57.2	58.0
Ursus	29.7	28.4	29.1	15.1	14.1	14.6	66.2	62.5	64.4
Zentaur	24.9	24.3	24.6	12.4	11.1	11.8	52.9	52.3	52.6
Means	27.6	26.6	27.1	13.5	12.6	13.0	58.6	56.6	57.6
LSD 5%5	V:1.02	Y:0.6	V×Y:1.3	V:0.7	Y:0.5	V×Y:0.9	V:2.4	Y:1.7	V×Y:2.1
	Yield (kg/da)			Dry matter content (%)			Dry matter yield (kg/da)		
Rekord	13.935	12.755	13.345	13.2	14.2	13.7	1.839	1.811	1.828
Rota	16.975	15.951	16.463	14.1	14.9	14.5	2.393	2.377	2.387
Ursus	19.958	18.661	19.309	15.4	16.4	15.9	3.074	3.060	3.070
Zentaur	13.565	12.125	12.845	12.5	13.2	12.9	1.696	1.601	1.657
Means	16.108	14.873	15.490	13.8	14.7	14.2	2.223	2.186	2.200
LSD 5%5	V:471	Y:261	V×Y:655	V:0.2	Y:0.3	V×Y:0.4	V:0.24	Y:0.13	V×Y:0.36
	Crude protein ratio (%)			Sugar ratio (%)			Wet weight loss (%)		
Rekord	8.06	8.52	8.29	5.14	5.41	5.28	23.5	21.9	22.7
Rota	8.51	8.91	8.71	5.19	5.61	5.40	24.8	22.8	23.8
Ursus	9.02	9.58	9.30	6.11	6.58	6.35	26.9	25.1	26.0
Zentaur	8.04	8.16	8.10	5.06	5.15	5.11	22.6	21.1	21.9
Means	8.41	8.79	8.60	5.38	5.69	5.53	24.5	22.7	23.6
LSD 5%	V:0.51	Y:0.61	V×Y:0.78	V:0.28	Y:0.32	V×Y:0.41	V:0.5	Y:0.6	V×Y:1.2

(V: Varieties, Y:Year, V×Y: Varieties × Y)

Table 4. Data on leaf properties obtained in the study

Plant Varieties	Leaf Properties								
	Length (cm)			Width (cm)			Yield (kg/da)		
	1. Year	2. Year	Mean	1. Year	2. Year	Mean	1. Year	2. Year	Mean
Rekord	51.3	49.4	50.4	17.6	17.2	17.4	1.975	1.825	1.900
Rota	56.3	54.1	55.2	19.9	18.8	19.4	2.388	2.195	2.292
Ursus	63.5	58.4	61.0	21.6	20.1	20.9	2.685	2.485	2.585
Zentaur	50.2	48.1	49.2	17.2	16.7	17.0	1.898	1.725	1.812
Means	55.3	52.5	53.9	19.1	18.2	18.6	2.237	2.058	2.147
LSD % 5	V:0.38	Y:0.27	V×Y:0.53	V:0.27	Y:0.19	V×Y:0.32	V:265	Y:161	V×Y:287
	Dry matter content (%)			Dry matter yield (kg/da)			Crude protein ratio (%)		
Rekord	12.2	13.1	12.7	241.0	239.1	240.0	21.9	21.2	21.6
Rota	13.2	14.2	13.7	315.2	311.7	313.5	22.8	22.1	22.5
Ursus	13.3	14.6	14.0	357.1	362.8	360.0	23.8	23.2	23.5
Zentaur	12.1	12.9	12.5	229.7	222.5	226.1	21.6	21.0	21.3
Means	12.7	13.7	13.2	284.0	281.9	283.0	22.5	21.9	22.2
LSD % 5	V:0.33	Y:0.24	V×Y:0.48	V:18.8	Y:764	V×Y:38.8	V:0.24	Y:0.13	V×Y:0.36

Sugar content in beets is directly proportional to the low rainfall, high temperature, and tuber dry matter content, the high altitude of the growing place and therefore the high day-night temperature difference. It is reported that the carbohydrates stored in the tuber during the day as a result of photosynthesis will not be lost when night temperatures drop to 6-7 °C and the sugar content is high (Acikgoz, 2021; Akyildiz, 1986; Ergul, 1988; Genckan, 1983; Salisbury & Ross, 1992). The fact that the altitude of the region where the research was conducted is low (31 m) and the temperature difference between day and night is less than in high altitude regions, caused the sugar rate to remain partially lower.

The results are lower than the data of Geren (1996) and Oz (1997), but close to those of Adiyaman (2003) and Yilmaz (2018).

Tuber weight loss in storage (%)

The obtained weight loss data are presented in Table 3. Among the varieties, the highest loss was determined in the Ursus variety with 26.0%, and the least loss was determined in the Zentaur variety with 21.9%. Loss rates in the first year were higher than in the second year. In terms of variety × year interactions, losses were high in parallel with the high tuber yield of the Ursus variety in both years.

One of the most important issues is that the entire product harvested for fodder beet cannot be consumed immediately, and therefore it must be preserved throughout the winter and until mid-spring. Storage can be done in open areas or in closed areas. No matter how the storage is done, a certain amount of yield loss is inevitable due to the tubers' ability to breathe, even if only slightly. In order to

reduce the loss, the open siled product should be covered with material that will not cause sweating, and in closed environments, the temperature should be low to minimize the respiration rate of the tubers. As temperature increases in storage, product loss also increases (Akyildiz, 1986; Ergul, 1988; Genckan, 1983; Soya et. al., 1997). An average loss of 23.6% (21.1-26.9) was determined for products stored in a closed warehouse without heating for 175 days.

The results are slightly less than the 27.3% loss of Adiyaman (2003) and Yilmaz (2018), who studied under Adapazarı conditions, and this shows that the storage conditions are appropriate.

Leaf Properties

Leaf length (cm)

Leaf length (including petiole) values taken from the leaves below the top leaves are given in Table 4.

In both years (63.5 and 58.4 cm), the longest average (61.0 cm) leaf length was taken from the Ursus variety, and the shortest tuber length was taken from the Zentaur variety.

While the leaf length data obtained in the study is compatible with the values reported by Albayrak & Yüksel (2009), it is higher than the values of Kokten & Ozdemir (2020).

Leaf width (cm)

Average data taken from the widest parts of the leaves below the uppermost leaves are given in Table 4. In both years and on average, the longest leaf length was taken from the Ursus variety, and the shortest tuber length was taken from the Zentaur variety.

While leaf width data obtained in the study is compatible with the values reported by Albayrak & Yüksel (2009), it is higher than the values of Kokten & Ozdemir (2020).

Herbage yield (kg/da)

Herbage yield values, one of the most important yield indicators, are presented in Table 4. According to the variety averages, the highest yield was obtained from the Ursus variety with 2.585 kg/da, and the lowest yield was from the Zentaur variety with 1.812 kg/da.

The first year data of the research is higher than the second year. In terms of variety × year interactions, the Ursus variety gave the highest yield (2.685 kg/da) in the first year.

It is reported that high temperature and rainfall values encourage the plant to make more assimilation and provide more leaf yield (Acikgoz, 2021; Akyildiz, 1986; Ergul, 1988).

From planting to harvest in the 1st and 2nd years of the research; precipitation of 388.0-248.5 mm, average temperature of 20.1-20.3 °C, and relative humidity of 73.8-71.4% were calculated. The long-term average of the same period is 235.6 mm, 20.4 °C, and 68.7%, in the same order. In this situation; the rainfall and relative humidity of both years in which the experiment was conducted were higher than the long-term average, and the average temperatures were close but slightly lower. In this case, it is natural that the efficiency is high.

The research results are compatible with the results of some research (Abou-Deya, 1991; Cetin & Ozhan, 1992; Geren, 1996; Voighlander & Jacob, 1987; Yilmaz, 2018), and higher than the results of some research (Adiyaman, 2003; Avcioglu & Sabanci, 1993; Cetin, 1998; Kokten & Ozdemir, 2020; Soya et. al., 1997).

Leaf dry matter content (%)

The results obtained by weighing and proportioning the dried samples are given in Table 4. The highest dry matter ratio was determined in the Ursus variety with 14.0%. In terms of years, the 2nd year data is higher than the 1st year data by 13.7%. In terms of variety × year interactions, Ursus varietty gave the highest rate with 14.6% in the second year. It is reported that the dry matter ratio of fodder beet leaves does not differ much according to the varieties and, is on average 12% (Sedlmayr, 1966).

The average 13.2% dry matter ratio obtained confirms Sedlmayr (1966) is similar to the findings of Adiyaman (2003), Geren (1996), and Yilmaz (2018), and is lower than the findings of Abou-Diya (1991) and Ergul (1988).

Leaf dry matter yield (kg/da)

The results are given in Table 4. The highest dry matter yield was obtained from the Ursus variety, both in terms of variety and variety × year interactions. In terms of years, the first year data is higher than the second year data.

The research findings are consistent with Geren (1996) and Yilmaz (2018), lower than Bartholomaeus (1988), and higher than Adiyaman (2003), and Kokten & Ozdemir (2020).

Leaf crude protein ratio (%)

The data obtained according to the analysis results are presented in Table 4. The highest rate was obtained from the Ursus variety, with 23.5%. In terms of years, the first year data (22.5%) is higher than the second year data (21.9%). In terms of variety × year interactions, Ursus variety gave the highest rate in both years.

The obtained rates are consistent with the data of Abou-Diya (1991), Cetin (1998), and Yilmaz (2018), and are higher than the results of Ergul (1988) and, Kokten & Ozdemir (2020).

Discussion and Conclusion

From the results obtained from the research, tuber yield, leaf yield, dry matter ratio, and yield, which are the most important yield characteristics for fodder beet, are the prominent features in evaluation both without and with storage.

It is reported that the average tuber yield of forage beet varieties is 5-20 tons/da, the leaf yield is 1-4 tons/da, and the dry matter rate is between 8-28% (Acikgoz, 2021; Akyildiz, 1986; Anonymous, 2002; Cristiansen-Weniger et. al., 1979; Cetin, 1998; Genckan, 1983; Oz, 1997; Soya et. al., 1997; Voighlander & Jacob, 1987).

The average tuber yield of the four varieties used in this research is 15.490 kg/da (12.125 - 18.958 kg/da), the leaf herbage yield is 2.147 kg/da (1.725 - 2.685 kg/da) and the dry matter ratio values are 13.2% (12.1-14.6%).

As can be seen from the data obtained, the performances of the varieties tested were above average values than the results of many studies (Abou-Deya, 1991; Adiyaman, 2003; Anonymous, 2002; Avcioglu & Sabanci, 1993; Bartolomaeus, 1988; Cetin, 1998; Cetin & Ozhan, 1992; Elmali, 1998; Geren, 1996; Kokten & Ozdemir, 2020; Manga, et. al., 1997; Oz, 1997; Ozdemir & Kokten, 2020).

The reason for this is that the climatic conditions of the research area, especially rainfall and temperature, are suitable for fodder beet cultivation (Acikgoz, 2021; Akyildiz, 1986; Genckan, 1983; Sedlmayr, 1966; Senf, 1961). Because, according to the long-term averages of the research area; the amount of precipitation was determined to be 685.9 mm, the average temperature was 14.7 °C and the relative humidity was 74.1%. When all the data are examined, it is seen that the 1st year data is higher than the 2nd year data. This is because the rainfall amount in the 1st year (388 mm) was higher than the 2nd year (248.5 mm).

When the data obtained at the end of the research are evaluated as a whole, the average data of all varieties used in the research are within the reported and recommended limits for the fodder beet plant (Acikgoz, 2021; Akyildiz, 1986; Genckan, 1983; Sedlmayr, 1966; Senf, 1961). Although the data from of all varieties were positive, as a result of the statistical analysis, the highest and most positive data were obtained from the Ursus variety. Fodder beet, which has a very high yield, is of great importance in filling the gap in quality forage for animals. For this reason, research on the subject; more clear and descriptive results should be achieved by using more varieties, different planting combinations, different fertilizer doses, and different soil types.

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Determination of Antifungal Activities on Some Plant Extracts on *Alternaria alternata*

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ABSTRACT

To increase yield and quality in agricultural production, it is necessary to perform management against diseases and pests. *Alternaria*, which causes several diseases in many economically important plants, is the most common species and widely distributed in nature. One of the important species reported in sweet cherry in recent years is *Alternaria alternata*. Many studies have emphasized the necessity of effective control with *Alternaria* species and examined the use of environmentally friendly methods against fungal diseases. In recent years, the use of plant extracts has increased due to their antimicrobial properties. Antifungal effects of *Datura stramonium* L., *Vitex agnus-castus* L., *Xanthium strumarium* L., *Capsella bursa-pastoris* L., *Convolvulus arvensis* L., *Viscum album* L., *Echinophora tenuifolia* L. *subsp. sibthorpiana* (Guss.) Tutin, *Amaranthus retroflexus* L., *Chenopodium album* L., *Tribulus terrestris* L., *Solanum nigrum* L., *Nerium oleander* L., *Circium arvense* (L.) Scop. and *Brassica oleracea* L. aqueous extracts were determined against *Alternaria alternata*. At the end of the 7-day incubation period, the mycelial growth of the fungi was measured and the antifungal effect of plant extracts was determined. As a result, the extracts were determined to inhibit mycelial growth compared to control. The plant water extracts used in the study were determined to inhibit the mycelial development of the pathogen by 20.20% to 77.12%. It is considered that different solvents and concentrations should be addressed to guide further studies. It was also concluded that potential plant species that may show anti-fungal properties should be evaluated.

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Introduction

Plants are the main source of food, fiber, forage, drugs, and many other useful products for humanity. Humans use different parts of plants such as roots, stems, leaves, fruits, flowers, and seeds to meet their food needs. Various insects, bacteria, viruses, fungi, and other pests cause harm decreasing yield in the several phases of the development of plants. It has been reported that more than 800 million people in developing countries experience food shortages and at least 10 percent of food crops are lost due to plant diseases (Strange & Scott, 2005). Fungi have the greatest impact in terms of disease and loss of crop production compared to other plant parasites. There are about 10.000 fungus species causing disease in plants and these fungi may cause losses not only in the growth period of plants but also in their harvest and storage periods. Harvest loss due to fungal diseases is about 12 % in all the products in the developing countries in the world. *Alternaria* species are the most commonly seen species in above-ground parts and they spread around nature broadly (Lopes & Martins, 2008). It also includes pathogenic saprophytic species that cause putrefaction both in the field and in the post-harvest period and lead to significant economic losses for the product and food industry (Logrieco et al., 2009). Alternaria is a widespread genus of fungi comprising more than 300 species, commonly found in soil and organic matter (Bessadat et al., 2021; Woudenberg et al., 2013). This genus contains saprophytic, endophytic, and pathogenic species. Several different secondary metabolites can be produced by *Alternaria* spp. blight disease, caused by *Alternaria* species, causes an average yield loss of 32-57 %. The members of the *Alternaria* genus such as *A. alternata, A. solani, A. porri, A. dauci, A. helianthi, A. carthami* and *A. makrospore* cause various diseases in different hosts (Chen et al., 2018; Kaya & Zorba 2021; Rotem, 1994). In sweet cherry cultivation, which is economically important in the world and Türkiye, fungal pathogens cause serious threats and cause significant crop losses. In recent years, the presence of *Alternaria alternata* in sweet cherries has been reported in Greece (Thomidis & Tsipouridis, 2006), China (Ahmad et al., 2020; Chethana

et al., 2019; Zhao & Liu, 2012), Italy (Wagas et al., 2023) and Türkiye (Şimşek et al., 2022). Many studies have emphasized the necessity for effective control of *Alternaria* species, which cause several diseases in many economically important cultivated plants, and various studies have been conducted on the use of environmentally friendly methods in controlling fungal diseases (Meena et al., 2020; Yadav et al., 2020).

The various treatments such as fungicides, antagonist organisms, and crop rotation are widely used in the control of plant diseases (Choudhary et al., 2004; Pineda, 2001). In addition, natural fungicides obtained from allelopathic and medicinal plants have become widespread all over the world as alternatives to other control methods due to their environmentally friendly and economic properties. Many researchers are currently working on effective natural products that can replace synthetic pesticides for the control of diseases.

These methods may be sorted as the use of antagonistic microorganisms, the use of plant extracts, or essential oil components and their derivatives. Several studies have shown that certain plant extracts can serve as a bio-pesticide source, effectively preventing the development of plant pathogens, and reducing harm to both human health and the environment. The existence of anti-fungal components in some plants is accepted to be an important factor in controlling some plant diseases (Tapwal et al., 2011). Although there is an increasing interest in the use of the extracts obtained from plants in controlling plant diseases, only 2.400 plant species have been screened among more than 250.000 plant species in terms of antimicrobial effect (Khafagi & Dewedar, 2000; Oluwalana & Adekunle, 1998; Oluwalana et al., 1999). As herbal pesticides are local, cheap, anti-toxic, and easily biodegradable, they provide significant advantages compared to synthetic fungicides (Akinbode & Ikotun, 2008; Bandara et al., 1989; Harlapur et al., 2007; Maji et al., 2005; Manoharachary & Gourinath, 1988; Nduagu et al., 2008; Srivastava & Lal, 1997; Yasmin et al., 2008). Plant metabolites and phytopharmaceuticals are considered as one of the alternative methods to control plant diseases (Varma & Dubey, 1999). As resistance to synthetic fungicides increases and residue levels rise, the use of natural products to control fungal diseases is considered one of the alternatives (Gurjar et al., 2012). The most important factor in weeds maintaining their vitality over time is that they are resistant to the pests and pathogens in their environment. They can therefore be used as a potential source of antimicrobial compounds. In recent years, interest in plant-based fungicides has been increasing due to their environmentally friendly properties (Abdessemed et al. 2021; Dwivedi & Singh, 1998; Karnwal & Singh, 2006). The inhibitory effect of plant extracts on pathogens has been demonstrated, and many higher plants and their compounds have proven successful in controlling plant diseases, without harm or toxicity (Dethoup et al., 2018, Kokkrua et al., 2020). In contrast to chemical fungicides, these extracts are harmless and non-phytotoxic (Alam et al., 2002; Charudattan et al., 2000; Dubey, 1991; Glare et al., 2012; Singh et al., 1986).

Plants can synthesize aromatic secondary metabolites such as phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins, and coumarins (Cowan, 1999). Especially plant extracts, which play an important role in the control of phytopathogens, have been used in many ways. The levels of phenolic compounds, plant pigments, and other chemical compounds in natural extracts have a synergistic effect on plant growth and are also effective in controlling many fungi. Because of these properties, plant extracts play an important role in preventing many pathogens that cause plant diseases (Nwachukwu & Umechuruba 2001). For this purpose, the study examined the antifungal effects of some plants and weeds against *A. alternata*, an important plant pathogen, under *in-vitro* conditions.

Materials and Methods

Plant Material

In the study, the aqueous extracts prepared from the plants *Datura stramonium* L., *Vitex agnus-castus* L., *Xanthium strumarium* L., *Capsella bursa-pastoris* L., *Convolvulus arvensis L.*, *Viscum album* L., *Echinophora tenuifolia* L. subsp. *sibthorpiana* (Guss.) Tutin, *Amaranthus retroflexus* L., *Chenopodium album* L., *Brassica oleracea* L., *Solanum nigrum* L., *Tribulus terrestris* L. *Nerium oleander* L., and *Cirsium arvense* (L.) Scop. were used (Table 1).

Table 1. The plants and plants part used in the study

Scientific name	Common name	Used part	Family
Capsella bursa-pastoris L.	Shepherd's purse	Above ground parts	Brassicaceae
Datura stramonium L.	Jamestown weed	Leaf	Solanaceae
Vitex agnus-castus L.	Chaste tree	Seed	Lamiaceae
Convolvulus arvensis L.	Field Bindweed	Above ground parts	Convolvulaceae
Viscum album L.	Mistletoe	Leaf	Loranthaceae
Viscum album L.	Mistletoe	Stem	Loranthaceae
Xanthium strumarium L.	Cocklebur	Leaf	Asteraceae
Echinophora tenuifolia L. subsp. *sibthorpiana* (Guss.) Tutin.	Turkish pickling herb	Above ground parts	Apiaceae
Amaranthus retroflexus L.	Pigweed	Leaf	Amaranthaceae
Chenopodium album L.	Lamb's quarters	Leaf	Amaranthaceae
Cirsium arvense (L.) Scop.	Creeping thistle	Above ground parts	Asteraceae
Solanum nigrum L.	Nightshade	Leaf	Solanaceae
Nerium oleander L.	Oleander	Leaf	Apocynaceae
Tribulus terrestris L.	Bullhead	Above ground parts	Zygophyllaceae
Brassica oleracea L.	Cabbage	Cabbage outer leaf	Brassicaceae

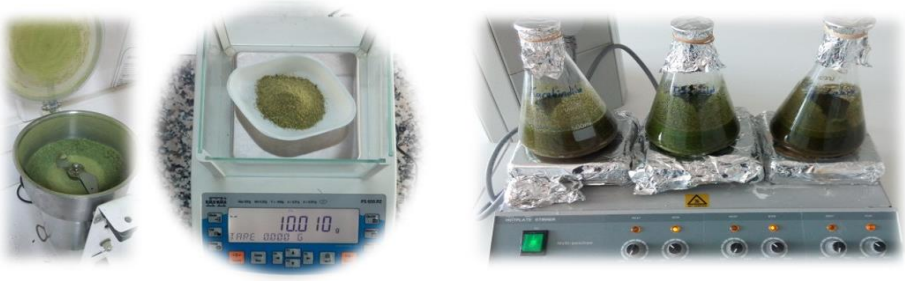

Figure 1. The preparation process of some plant extracts

Figure 2. Transfer of each plant extract to PDA

Fungal Material

A. alternaria (Accesion number MW509978) B1-3 isolate obtained from diseased sweet cherry leaves were used as the fungal material of the study. In the study, 7 day cultures of the pathogen developed in PDA medium at 25+2ºC were used.

Method

Healthy plants were collected and brought to the laboratory. They were cleaned with tap water to remove soil and washed with sterile distilled water. All the plants were dried in an oven at 50 °C until they became completely crispy (Bussaman et al., 2012). The dried leaves were ground and were kept at room temperature in glass jars with screw lids under laboratory conditions.

Preparing plant extracts and Determining the Antifungal Effect on Mycelial Growth

The ground plant parts were diluted with sterile distilled water at the rate of 1/15 (10 gr plant/150 ml distilled water) (Türküsay & Onoğur 1998) and left for extraction on shaker at room temperature for 2 hours (Figure 1). The extracts were filtered in 2-4 folded sterile cheesecloth. The stock extracts obtained from each plant used in the study and the PDA growth medium were sterilized for 15 minutes at 121 °C in an autoclave. The sterilized extracts were added to the growth medium at the rate of (1:1) and the mixture was placed into petri dishes (90 mm diameter), 15 ml for each. Petri dishes were kept for 1 night at room temperature for the environment to be ready for fungal inoculation (Figure 2). Discs obtained from pathogen cultures grown for 7 days in PDA growth medium using a 5 mm cork borer were placed in the centre of each petri, one disc in each petri, and left to incubate at 25+2ºC. The control Petri dishes contained PDA growth medium with sterile distilled water. The test was conducted with 5 repetitions. Colony diameter was measured in perpendicular directions by Benjilali et al (1984), and using a digital caliper and mycelial growth of fungus in determining the anti-fungal effect of the plant extracts was assessed. The % inhibition of the plant extracts on mycelial growth was calculated. The formula used in the calculation is stated below. The % inhibitive effect of the plant extracts on mycelial growth compared to the controls, was calculated by the following formula (Mohana & Raveesha, 2007).

I= C – A / C×100
I= Inhibition (%)
C= Colony diameter in control petri dish (mm)
A= Colony diameter in application petri dish (mm)

Statistical Analysis

To determine the differences between the treatments in the tests, we conducted a significance analysis of variance (ANOVA) and compared the means using the DUNCAN test. Statistical analysis was evaluated by using the SPSS 23.0 packaged software.

Results and Discussion

Alternaria causes leaf diseases with economic significance on a wide range of host plants including cereals, vegetables, fruit, ornamentals, and forest trees. The effects of the water extracts obtained from *Datura stramonium*, *Vitex agnus- castus*, *Xanthium strumarium*, *Capsella bursa-pastoris*, *Convolvulus arvensis*, *Viscum album*, *Echinophora tenuifolia* L. subsp. *sibthorpiana* (Guss.) Tutin, *Amaranthus retroflexus*, *Chenopodium album*, *Tribulus terrestris*, *Solanum nigrum*, *Nerium oleander*, *Circium arvense*, and *Brassica oleracea* plants on the mycelial growth of *A. alternata* under *in-vitro* conditions were determined (Table 2). *Brassica oleracea* L. (cabbage outer leaf) came out on top with an inhibition rate of 77.12 % and was statistically different from the other extracts (Figure 3). A study reported that plant extracts obtained from six plant species belonging to six different families (Alliaceae, Brassicaceae, Lythraceae, Lamiaceae, Solanaceae and Verbenaceae) showed significant antifungal effects against *F. oxysporum* f.sp. *lycopersici* and completely prevented conidial germination (Rongai et al., 2015). *N. oleander* and *D. stramonium* were

placed near the top with inhibition rates of 45.47 % and 43.15 %, respectively. *Allium sativum* L. and *Eucalyptus occidentalis* L. extracts completely inhibited the pathogen at 5% and 10% concentrations against *A.brassicae* causing Alternaria blight on mustard, followed by *Polyanthi longifolia* (90.77% and 100%), *Ocimum sanctum* L (87.44% and 100%), *Datura stramonium* L (85.09% and 100%), *Azadirachta indica* L (82.44% and 100%). Similarly, it was reported that neem and chilli plant extracts were highly effective against *A. brassicicola* Alternaria leaf spot disease in cabbage at 15% and 25% concentrations and inhibited mycelial growth by 68 % at 25% concentration (Gupta et al., 2019). Hassanein et al. (2008) reported similar results against *A. alternata*, which causes early blight in tomato. Wszelaki & Miller (2005) also found that garlic extracts significantly reduced the intensity of leaf blight disease in tomato. Similarly, Panchal & Patil (2009) conducted in vitro studies to test the effectiveness of garlic, turmeric, and neem extracts at a 10% concentration against *A. alternata*. The results showed that garlic clove extract was highly effective in reducing Alternaria fruit rot of tomato, followed by turmeric and neem extracts. The lowest inhibition rate was observed in *T. terrestris, C. arvense* and *A. retroflexus* treatments among the plant extracts used. With the water extracts obtained from *V. album* (stem), *S. nigrum, Vitex agnus-castus, C.bursa-pastoris*, the mycelial growth of *A. alternata* was inhibited approximately at the rates of 32.78-37.33 % and this inhibition rate was determined to be about 45 % for *D. stramonium* and *N.oleander*. The inhibition rate in the other plants used varied between 20 % and 26 %. The control treatment was different from all the other treatments and the plant extracts used inhibited the mycelial growth of *A. alternata* at different rates. Sharma et al. (2021) reported *Allium sativum* extract was found to inhibit mycelial growth of *Alternaria alternata* by 90.11%, 100% and 100% at 5%, 10% and 15% concentrations respectively, followed by *Azadirachta indica* leaf extract (79.45%, 83.60% and 88.22%). *Alstonia scholaris* leaf extract inhibited mycelial growth of the pathogen less than the control at 5%, 10% and 15% concentrations by 36.22%, 40.33% and 47.77%, respectively. Cherkupally et al. (2017) reported that *D. stramonium* water extracts inhibited the mycelial growth of *R. solani* and *Fusarium oxysporum* f.sp. *melongenae* by 72% and 61.1% at 20% (highest) concentration, respectively. The mycelial growth of *M. phaseolina* was inhibited by 0.0%, 27.7% and 57.7% at 5%, 10% and 20% concentration, respectively. Based on the results obtained, it was observed that the plant extracts can inhibit the *in vitro* colony growth of *A. alternata* isolated from sweet cherry leaf at high or low rates.

In the study assessing the anti-fungal effects of the water extracts prepared from leaves of some annual and perennial weeds and cultivated plants against *A. alternata, A. solani, Botrytis cinerea,* and *Drechslera sorokiniana* under *in-vitro* conditions, it was observed that *Hedera helix* leaf extract inhibited spore germination and colony growth at the highest rate. It was stated that *D. stramonium* extract inhibited *A. alternata* sporulation density at the rate of 41 % and the colony growth at the rate of 15 % (Türküsay & Onoğur, 1998). It was stated in another study that *D. stramonium* inhibited the mycelial growth of *A. alternata* at the rate of 19.66 % (Ögüt Yavuz et al., 2018).

The effect of *D. stramonium*, included in the present study, on the colony growth of *A. alternata* was recorded to be 43 %. The difference in the inhibition rate is considered to be associated with the plant collection period, the plant organ used and the amount of extract used in the environment. Alkaloids such as tporane (atropine), hyoscyamine, and scopolamine included by *D. stramonium* and their amounts may have a role in the anti-fungal effect. In general, there may be more alkaloid in certain organs of plants (such as root, shell, leaf, fruit, seed). As a result of the study in which the leaf water extracts of *D. stramonium*, *D. innoxia*, *D. metal* and *D. ferox* at different concentrations on mycelial growth of *Alternaria solani* were assessed; 20 % concentration of *D. stramonium* inhibited the mycelial growth of *A. solani* (at the rate of 88 %) and this is in parallel with the results of the present study (Jalander & Gachande, 2010).

Table 2. The effects of different plant extracts on the mycelial growth of *Alternaria alternata* (%)

Plant Extracts	*Alternaria alternata*	
	Colony diameter* (mm)	% Effect
Brassica oleracea L.	16.94 I	77.12
Nerium oleander L.	40.38 H	45.47
Datura stramonium L.	42.10 GH	43.15
Capsella bursa-pastoris L.	46.41 FG	37.33
Viscum album L. (stem)	49.78 EF	32.78
Vitex agnus-castus L.	50.67 DEF	31.57
Solanum nigrum L.	50.72 DEF	31.51
Viscum album L. (leaf)	51.60 CDEF	30.32
Xanthium strumarium L.	54.77 BCDE	26.04
Chenopodium album L.	55.59 BCD	24.93
Echinophora tenuifolia L. subsp. *sibthorpiana* (Guss.) Tutin.	56.51 BC	23.69
Convolvulus arvensis L	56.82 BC	23.27
Amaranthus retroflexus L.	57.11 B	22.88
Cirsium arvense (L.) Scop.	59.03 B	20.28
Tribulus terrestris L:.	59.09 B	20.20
Control	74.05 A	0

* The means belonging to different letters in the same column are different at the significance level of P<0.05 according to DUNCAN.

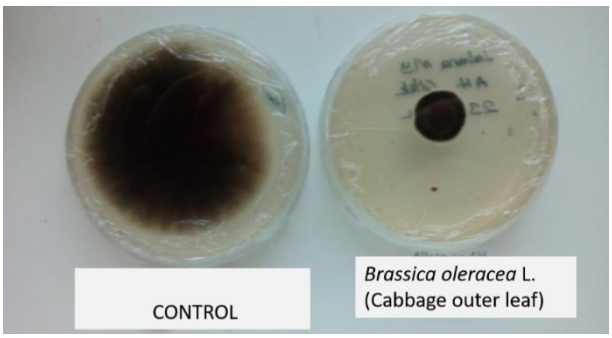

Figure 3. Effect of cabbage outer leaf water ectract on mycelial development of *A. alternata*

It was stated that the spore germination of *A. brassicae* isolated from cauliflower leaves was inhibited by using the *Canna indica*, *Convolvulus arvensis*, *Ipomoea palmata*, *Cenchrus catharticus*, *Mentha piperita*, *Prosopis spicigera*, *Allium cepa*, *A. sativum*, *Lawsonia inermis*, *Argemone mexicana*, *D. stramonium* and *Clerodendron inerme* extracts. Although the anti-fungal effect of *A. retroflexus* extract against *A. alternata* was lower compared to the other treatments, there was an effect of about 22 % compared to the control (Sheikh & Agnihotri, 1972). In the study examining the antifungal effects of *Orobanche ramosa*, *Viscum album* and *Cuscuta campestris* against *Alternaria solani, Monilinia fructigena, Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL), it was stated that all the plant extracts used had a significant anti-fungal effect and the inhibition rate in *V. album* in 20 mg ml^{-1}doses of the extracts was stated to be 60 % against *A. solani* (Şin et al., 2017). In the present study, it was determined that the inhibition rate obtained with *V. album* (stem-leaf) was approximately 30 %-33 %. In the present study, an inhibition rate of 45.47 % was observed in *N. oleander* water extract Singh & Srivastava (2014), and the highest inhibition rate against *A. alternata* in methanol and water extract under in-vitro conditions was obtained with oleander treatment among different plants (*Partizyum hysterophorus*, *Vernonia amygdalina*, *Eucalyptus camaldulensis*, *Nerium oleander*, *Lantana camara* and *Ocimum sanctum*) and at different concentrations (5 %, 10 %, and 20 %). This indicated that methanol extracts were more effective against *A. alternata* compared to water extracts. It was reported that the mycelial growth was significantly inhibited against *A. alternata* with the increasing concentrations of the extracts. The effects of different sterilization techniques on the anti-fungal activities of root and leaf extracts of (*Urtica dioica* L.) stinging nettle at different concentrations (2.5, 5.0, 10.0, 20.0, 40.0 %) in mycelial growth and spore germination of *Alternaria solani* were examined. The highest inhibition rate of the extracts sterilized in the autoclave for mycelial growth was determined to be 7 % in root extracts and the root extracts sterilized with filtration technique affected the mycelial growth and spore germination at the rates of (75 % and 38 %, respectively) (Nabrdalık & Grata, 2015). The antifungal activity of ethanol extracts of nettle (*Urtica dioica* L.), colocynth (*Citrullus colocynthis* L. Schrad), konar (*Ziziphus spina-christi* L.) and oleander (*Nerium oleander* L.) flower parts were investigated against *Alternaria alternate*, *Fusarium oxysporum*, *Fusarium solani* and *Rizoctonia solani* under in vitro conditions. The extracts showed antifungal activity against these pathogens. Among the plants, nettle and colocynth were most effective against *A. alternate* and *R. solani*, while oleander showed the best inhibition effect against *F. oxysporum* and *F. solani*. It was reported that the extracts used in the study can be used as an alternative to synthetic chemicals in the control of fungal diseases in plants (Hadizadeh et al., 2009).

Vitex agnus-castus L. included in the present study inhibited the mycelial growth of *A. alternata* at the rate of 31.57 % compared to the control. The research shows that the methanol extract of *Vitex agnus-castus* has an inhibitory effect on the mycelial growth of *A. solani*. Gradually increasing doses of the extract resulted in a corresponding increase in inhibition levels of mycelium development in *A. solani*, with values of 27.73%, 32.98% and 40.08% respectively (Yılar et al., 2015).

Conclusion

In conclusion, it was determined that the water extracts prepared from *C. bursa-pastoris, D stramonium, Vitex agnus-castus, C. arvensis, V. album, X. strumarium, Echinophora tenuifolia, A. retroflexus, C. album, B. oleracea, S. nigrum, N. oleander, C. arvense* plants had the anti-fungal effects against *A. alternata* isolated from sweet cherry leaf under *in-vitro* conditions. The anti-fungal effect of cabbage outer leaf, oleander, and jimsonweed weed water extracts was placed near the top with its high inhibition rate. Compared to the control, the other plant extracts inhibited the mycelial growth of *A. alternata* at different rates. In further studies, the activities of the plant/plants with the highest anti-fungal effect should also be assessed under *in-vivo* conditions. This study revealed hopeful results demonstrating that the extracts obtained from plants may be used as an alternative to synthetic pesticides in the control of plant diseases and it is considered to be a reference for further studies.

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A Comparative Analysis of Household Food Insecurity Status among Rice Farmers in Savanna and the Rainforest Agro-ecological Zones in Southwest States, Nigeria

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ABSTRACT

The study presents findings on comparative analysis of household food insecurity status among rice farmers in the Savanna and the Rainforest agro-ecological zones in Southwest States, Nigeria. Primary data were used and obtained through the administration of a well structured questionnaire. A multistage random sampling was used to select 577 rice farmers in the study area. Data were analyzed using descriptive statistics, household food insecurity access (HFIA) scale, household food insecurity access prevalence (HFIAP) scale, likert scale and linear regression model. The results revealed that majority of the rice farmer's fall within 31-50 years of age bracket, with household size of 5-8 persons, married, with farming experiences and have small farm size. The findings from average household food insecurity access scale scores in the Savanna and the Rainforest agro-ecological zones were 4.0 (mildly food insecure) and 5.2 (moderately food insecure) respectively. The results of HFIAP indicator revealed that about 39.1% and 33.5% of respondents were classified as food secure, 8% and 13.9% were mildly food insecure, 15.1% and 22.2% were moderately food insecure and 37.8% and 30.4% were severely food insecure in the Savanna and the Rainforest agro-ecological zones respectively. The major coping strategies adopted by the respondents against food insecurity include reduce the quantity of food consumed and eating but not satisfied. The linear regression model revealed that age, sex, years in school, farm size, household size, farming experience, rice farming experience and tenure system significantly affect household's food insecurity status. To transport from food insecure to food secure, age, sex, years in school, farm size, household size, farming experience, rice farming experience and tenure system alleviation policies are imperative.

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Introduction

Nigeria is the most populous nation in Africa with almost 186 million people in 2016 (UNICEF 2017). With a high fertility rate of 5.38 children per woman, the population is growing at an annual rate of 2.6 percent, worsening overcrowded conditions. By 2050, Nigeria's population is expected to grow to a staggering 440 million, which will make it the third most populous country in the world, after India and China (Population Reference Bureau, 2013). A scarcity of resources and land in rural areas has resulted in Nigeria having one of the highest urban growth rates in the world at 4.1 percent (Nigeria Federal Ministry of Health 2014). Currently, Nigeria ranks 145th out of 157 countries in progress toward meeting the Sustainable Development Goals (SDGs) (Sachs et al., 2017).Thus, the aforementioned situations in Nigeria as a country and its economy have the probability of making large number of the populace vulnerable to food insecurity in the country. In Nigeria, about 5.3 million people were food insecure in 16 states of the country (GRFC, 2019). In addition, not less than 70% of the Nigerian population is surviving on less than a dollar per day while food insecurity prevalence in the low income urban households and rural areas respectively stands at 79% and 71% (Akerele et al.; 2013).

In spite of availability of cultivable land area, the current level of demand for rice in Nigeria is about 5 million metric tonnes which is more than the quantity produced (2.2 metric tonnes) (Ajetomobi et al., 2010). Consumption of rice has already outpaced domestic production and as a result, Nigeria is the leading importer of rice in the world today, with an 8.2 percent share of imports in the global market (Gyimah-Brempong et al., 2016). Rice import represents more than 25% of agricultural imports and over 40% of domestic consumption (Ohaka et al., 2013). Despite the place of rice in contributing to the food supply in Nigeria, its production

is still put at 3.2 million tonnes (Babafada, 2003; Ohaka et al., 2013). This has shown to be far below the national requirement as over 600 million dollars' worth of rice is imported annually into the country (Adeoye, 2003; Ohaka et al., 2013; Raufu, 2014; Abdullahi 2012, Omofesho, 2010). This study therefore investigated comparative analysis of household food insecurity status among rice farmers in Savanna and the Rainforest agro-ecological zones in Southwest States, Nigeria. The specific objectives are to describe the socio-economic characteristics of rice farming households and analyse household food insecurity status of rice farmer's households by comparing their socio-economic characteristics.

Materials and Methods

Study Area

The study area was Southwest Nigeria comprising of Lagos, Ogun, Oyo, Osun, Ondo and Ekiti States. The six States lie between longitude 2°31[1] and 6°00[1] East and latitude 6°21[1] and 8°37[1] North with a total land area of 77, 818 km^2. The study area is bounded in the East by Edo and Delta states, in the North by Kwara and Kogi States, in the West by the Republic of Benin and in the South by the Gulf of Guinea. Two distinct (dry and wet) seasons are dominant in the study area in which subsistence and small scale farming are practiced (Odekunle et al., 2007).

The climate of the study area experiences a double rainfall maxima characterized by bimodal high rainfall peaks, with a short dry season and a longer dry season falling between and after each peaks. The mean annual rainfall is between 1200mm and 1500mm. Atmospheric temperature in Southwest, Nigeria is high throughout the year with an annual mean of 27° (BNRCC, 2011).

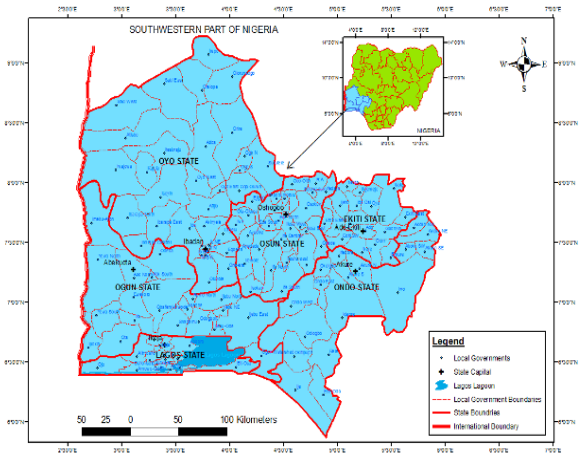

Figure 1.Map of the Southwest States, Nigeria
Source:Agboola and Olurin, 2003

Data and Sampling Procedure

Primary data for this study were collected in 2021 during rice production period through the use of a well-structured questionnaire administered through direct interviews to rice farming households in the study area. A multistage random sampling technique was used for selection of the respondents. The first stage involved a purposive selection of the two dominant agro-ecological zones (that is, Savanna and Rainforest agro-ecological zones) in the Southwest, Nigeria with about 32.5 million people (NPC, 2006). Ekiti and Oyo States belong mainly to Savanna dominated agro-ecological zone. While Ondo, Ogun and Osun States mainly belong to Rainforest agro-ecological zone. Lagos State was not included because of administrative reason (Otitoju, 2013). The second stage involved purposive selection of Ekiti, Ondo and Ogun out of the six States in Southwest Nigeria because of high rate of rice production (land under rice cultivation is about 2 million hectares) in the three States (Arimi 2014; Osabuohien et al., 2018).

The third stage involved purposive selection of six (6) Agricultural Development Programme (ADP) zones in the three States based on the predominance of rice farmers in these zones (Table 1). The fourth stage involved purposive selection of two (2) extension blocks from each Agricultural Development Programme (ADP) based on the predominance of rice farmers (Table 1) in these extension blocks, making twelve (12) extension blocks in all. At the final stage, respondents were randomly selected from each of the cells proportionate to the population size of the cells. In all, 225 and 352 rice farming households were sampled in the Savanna and Rainforest agro-ecological zones respectively (Table 1).

Analytical Framework

Descriptive Statistics

The data collected from the respondents were analysed using descriptive statistics such as frequency counts, percentages and mean. This tool was used to describe the socio economic characteristics of the respondents in the study area.

Household Food Insecurity Access Score (HFIAS) Model

Food security was measure by HFIAS and it was used to categorized respondents as food secure, mildly food insecure, moderately food insecure, or severely food insecure (Coates et al. 2007). The HFIAS was developed by the USAID Food and Nutrition Technical Assistance project (FANTA 2006) in an increasingly need to have a universally comparable and cost-effective measure of food security (Coates et al., 2007) and have been used in a similar studies by Gabriela and Manfred (2007) and Ibrahim et al. (2009).

The HFIAS module covers a recall period of 30 days, and consists of 18 questions that were grouped into two types of questions - nine "occurrence" and nine "frequency-of-occurrence" questions. The respondent is first asked if a given condition was experienced (yes, no or I don't know) and, if it was, then with what frequency (rarely that is, once or twice in the past four weeks, sometimes that is, three to ten times in the past four weeks or often that is, more than ten times in the past four weeks). The resulting responses were transformed into a continuous indicator and categorical indicator of food security respectively. When calculating as a continuous indicator, each of the nine questions is scored between 0-3, with 3 being the highest frequency-of-occurrence (often). The score for each is then added together. The total HFIAS range from 0 to 27 indicating the degree of insecure food access. While the HFIAP indicator (Table 2) was used to categorized households as food secure, mildly food insecure, moderately food insecure, or severely food insecure (Coates et al. 2007).

Table 1. Distribution of the Research Sample of Rice Farmers in Southwest, Nigeria

Agro-ecological zones	States	ADP Zones	Extension Blocks	Farming Community	Sampling Frame	Sampled Farmers
Savanah	Ekiti	Zone I	Aramoko	Aramoko	22	20
				Ora	20	19
				Ido-ile	18	17
			Igede	Ensure	19	18
				Awo	22	20
				Igbemo	19	18
		Zone II	Ikole	Ayedun	22	20
				Ipao	19	18
				Itapaji	22	20
			Oye	Ire	18	17
				Ilupeju	20	19
				Ayede	20	19
Rainforest	Ogun	Ilaro	Imeko	Iba	17	16
				Ijagba	16	15
				Fetedo	17	16
			Igbogila	Egwua	15	14
				Shangisha	16	15
				Igan-alade	16	15
		Abeokuta North	Abeokuta North	Ijale papa	16	15
				Anigbado	15	14
				Tibo-Akungun	16	15
			Ewekoro	Ipakodo	15	14
				Lukoti	17	16
				Ebidagba	16	15
Rainforest	Ondo	Ondo North	Akoko north	Ikaramu	22	20
				Ute	20	19
				Uso	18	17
			Ose/Owo	Ogbeese	22	20
				Owode	15	14
				Alayere	22	20
		Ondo Central	Okitipupa	Iju Odo	20	19
				Ikoya	15	14
				Ode aye	22	20
			Ileoluji	Uloein	25	23
				Ileoluji	20	19
				Bamikemo	22	20
Total	3	6	12	36	676	577

Source: Author' Construct, 2021.

Table 2. Household Food Insecurity Access Prevalence

HFIAP category
The Household Food Insecurity Access category for each household was calculated as follows:
HFIAP category = 1 Food Secure, 2=Mildly Food Insecure Access, 3=Moderately Food Insecure Access, 4=Severely Food Insecure Access
HFIA category = 1 if [(Q1a=0 or Q1a=1) and Q2=0 and Q3=0 and Q4=0 and Q5=0 and Q6=0 and Q7=0 and Q8=0 and Q9=0]
HFIAP category= 2if [(Q1a=2 or Q1a=3 or Q2a=1 or Q2a=2 or Q2a=3 or Q3a=1 or Q4a=1) and Q5=0 and Q6=0 and Q7=0 and Q8=0 and Q9=0]
HFIAP category= 3 if [(Q3a=2 or Q3a=3 or Q4a=2 or Q4a=3 or Q5a=1 or Q5a=2 or Q6a=1 or Q6a=2) and Q7=0 and Q8=0 and Q9=0]
HFIAP category = 4 if [Q5a=3 or Q6a=3 or Q7a=1 or Q7a=2 or Q7a=3 or Q8a=1 or Q8a=2 or Q8a=3 or Q9a=1 or Q9a=2 or Q9a=3]

Source: Coaste et al. 2006.

Linear Regression Model

Linear regression model was used to test the relationship between socio economic characteristics of the respondents and their food insecurity status. The model is stated thus:

$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_6X_6 + \varepsilon$

Y = Food Insecurity status of the respondents

X_1 = Age

X_2 = Sex

X_3 = Marital status

X_4 = Years in school

X_5 = Farm size

X_6 = Household size

X_7 = Extension service

X_8 = Farming experience

X_9 = Rice farming experience

X_{10} = Tenure system

X_{11} = Income

ε = error term

Results and Discussion

Table 3 revealed that about 80.5% and 75.6% of the rice farmers in the Savanna and the Rainforest agro-ecological zones respectively fall within 31-50 years of age bracket with an average age of the rice farmers in Savanna and Rainforest agro-ecological zones (SRAEZs) were 45.4 and 44.7 years respectively, implying that the rice farmers in these zones are in their economically active age. This was similar to the mean age of 49.8 years of crop farmers reported by Ogunniyi et al. *(*2021). Majority (77.8% and 83.8%) of the rice farmers in the SRAEZs respectively were male, indicating that most communities in the study area are traditionally patriarchal in nature. The results further revealed that about 26.2% and 28.4% of the respondents in the SRAEZs respectively had 6 and below years of formal education with an average year of schooling of 12 and 10 years respectively, implying a low level of education among rice farming households.

The findings also showed that majority of the respondents (57.3% and 54.8%) in the SRAEZs have household size of 5-8 persons with an average household size of 7 and 5 persons respectively, suggesting that rice farming households have relatively large members, which could possibly be available as family labor against short fall of hired labor. These results corroborate the findings that a relatively large household size (especially of working age) reduces the constraint on labour demand in production, processing, and marketing (Opondo et al., 2017). The result in Table 3 shows that majority (91.6% and 93.5%) of the rice farmers in the SRAEZs were married. It was revealed that about 56.5% and 45.5% of the rice farmers in the SRAEZs respectively had above 15 years of farming experience, suggesting that majority of the rice farmers are well knowledgeable about rice production in the study area.

Food Security Status of Rice Farmers in the Study Area

The responses from the nine questions of the HFIAS questionnaire was used to compute HFIAS score presented in Table 5. The HFIAS score was used to generate the minimum, maximum, average values and categorizes households into four levels of food insecurity. These four categories are food secure, mildly food insecure, moderately food insecure and severely food insecure. The household food insecurity score ranges from 0 to 27, with a high score indicating greater vulnerability to food insecurity.

Average Household Food Insecurity Access Scale

The findings of the food security status of the rice farmers compute from average HFIAS score in Table 4 revealed that the average scores measuring vulnerability to food insecurity of rice farmers in the Rainforest and the Savanna agro-ecological zones were 4.0 (mildly food insecure) and 5.2 (moderately food insecure) respectively with the minimum score was 0 and the maximum score was 27. The standard deviation values of 4.9 and 4.6 for Rainforest and the Savanna agro-ecological zones respectively which implies that there was a high variation between the individual score ranging from 0 to 27. However, for the total sample in the study, the average score HFIAS measuring vulnerability to food insecurity was 4.3 (moderately food insecure), the minimum score was 0 and the maximum score was 27. The standard deviation of 4.8 implied that there was also a high variation between the individual scores ranging from 0 to 27.

Table 3. Frequency Distribution of Respondents by their Socio-Economic Characteristics in the Savanna and the Rainforest Zones in Southwest, Nigeria

Variables	Savanna (n=225)		Rainforest (n=352)		Total sample (n=577)	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
Age (years)	Mean = 45.4		Mean = 44.7		Mean = 45.3	
≤ 30	5.0	2.2	21.0	6.0	26.0	4.5
31-40	78.0	34.7	83.0	23.6	161.0	27.9
41-50	103.0	45.8	183.0	52.0	286.0	49.6
51-60	29.0	12.9	60.0	17.0	89.0	15.4
> 60	10.0	4.4	5.0	1.4	15.0	2.6
Sex						
Female	50.0	22.2	57.0	16.2	107.0	18.5
Male	175.0	77.8	295.0	83.8	470.0	81.5
Education (years)	Mean = 12.1		Mean = 10.4		Mean = 11.8	
≤ 6	59.0	26.2	100.0	28.4	159.0	27.6
7-12	91.0	40.4	178.0	50.6	269.0	46.6
≥ 13	75.0	33.3	74.0	21.0	149.0	25.8
Marital Status						
Single	11.0	4.9	11.0	3.1	22.0	3.8
Married	206.0	91.6	329.0	93.5	535.0	92.7
Widow/Widower	8.0	3.6	12.0	3.4	20.0	3.5
Farm Size (ha)	Mean = 4.6		Mean = 3.7		Mean = 4.0	
≤ 2	11.0	49.3	192.0	54.5	290.0	50.3
2.1- 4	37.0	16.4	115.0	32.7	162.0	28.1
4.1 and above	76.0	33.8	45.0	12.8	124.0	21.5

Source: Computed from field data, 2021.

Table 4. Frequency Distribution of Respondents by their Socio-Economic Characteristics in the Savanna and the Rainforest Zones in Southwest, Nigeria

Variables	Savanna (n=225)		Rainforest (n=352)		Total sample (n=577)	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
Farming experiences (years)	Mean = 20.7		Mean = 16.3		Mean = 18.0	
≤ 5	21.0	9.3	21.0	6.0	42.0	7.3
6-10	57.0	25.3	97.0	27.6	154.0	26.7
11-15	20.0	8.9	74.0	21.0	94.0	16.3
> 15	127.0	56.4	160.0	45.5	287.0	49.7
Rice farming experiences (years)	Mean = 9.7		Mean = 8.7		Mean = 7.3	
less than 5	21.0	9.3	21.0	6.0	42.0	7.3
6-10	57.0	25.3	97.0	27.6	154.0	26.7
11-15	20.0	8.9	74.0	21.0	94.0	16.3
Above 15	127.0	56.4	160.0	45.5	287.0	49.7
Distance (km)	Mean = 7.5		Mean = 5.6		Mean = 6.4	
1-2	67.0	29.8	114.0	32.4	182.0	31.5
3-4	81.0	36.0	139.0	39.5	219.0	38.0
Above 5	77.0	34.2	99.0	28.1	176.0	30.5

Source: Computed from field data, 2021.

Table 5. Household Food Security Status of Rice Farmers in Savanna and the Rainforest Agro-ecological zones Southwest, Nigeria.

Scores	Savanna	Rainforest	Total sample
Minimum score	0.00	0.00	0.00
Maximum score	27.00	27.00	27.00
Mean	5.16	4.01	4.37
Standard deviation	4.96	4.67	4.79

Source: Computed from field data, 2021.

Table 6. Household Food Insecurity Access Prevalence (HFIAP) of Rice Farmers in Southwest, Nigeria.

Food Security Status	Savanna		Rainforest		Total Sample	
	Frequency	percent	Frequency	percent	Frequency	percent
FS	88	39.1	118	33.5	206	35.7
MFIA	18	8.0	49	13.9	67	11.6
MFI	34	15.1	78	22.2	112	19.4
SFI	85	37.8	107	30.4	192	33.3
Total	225	100.0	352	100.0	577	100.0

Less than or equal to 1= food secure (FS), between 1.1- 4 = mildly food insecure access (MFIA), between 4.1-6 = moderately food insecure (MFI) and greater than 6 = severely food insecure (SFI). Source: Computed from field data, 2021.

Household Food Security Levels

This section depicts the categorisation of household food security status of the rice farmers by using the HFIAP indicator which is a subset of HFIAS model (Table6). The HFIAP indicator was used to observe household food security; and food insecurity prevalence (Coates et al., 2007). In this study, the HFIAP indicator categorised rice farmers' households into four main levels of food security status (food secure, mildly, moderately and severely food insecure) depending on how rice farmers responded to the nine-frequency-of-occurrence questions (Table 6). Based on the HFIAP classification measure of food security, about 39.1% and 33.5% of rice farmers in the Rainforest and Savanna agro-ecological zones were classified as food secure respectively while the remaining 60.9% and 66.5% were food insecure in the study area respectively. Thus, the findings reveal that rice farming households in Rainforest agro-ecological zone were more food secure when compared with rice farming households' in Savanna agro-ecological zone (Table 9).The finding for the pooled sample shows that about 35.7% of the rice farming households' in Southwest was food secure while 64.3% were food insecure. This implies that only 35.7% of the household's member interviewed have access to safe and sufficient food and were not worry about food access. In order words, only 35.7% of the respondents rarely experienced anxiety about not having enough food and have a full meal three times in a day without food running out, in the past 30 days. The findings of food security status of the rice farmers in the Savanna and the Rainforest agro-ecological zones further revealed that 8% and 13.9% were mildly food insecure respectively. Thus, the findings reveal that households in Savanna agro-ecological zone were more mildly food insecure when compared with Rainforest agro-ecological zone. The finding for the pooled sample shows that about 11.6% of the rice farmers in Southwest were mildly food insecure. This implies that about 11.6% households were anxious about not having sufficient food. They usually consumed inadequate diet, or ate food that they did not prefer. However these households did not experience the three severe conditions of going a whole day without eating, going to bed hungry or running out of

food in the last 30 days. Furthermore, the findings of food security status of the rice farmers in the Savanna and the Rainforest agro ecological zones revealed that 15.1% and 22.2% were moderately food insecure. Thus, the findings reveal that respondents in Savanna agro-ecological zone were more moderately food insecure when compared with Rainforest agro-ecological zone. The finding for the pooled sample shows that about 19.4% of the rice farmers in Southwest were moderately food insecure. This implies that about 19.4% of the households do not have access to safe and sufficient food and they began sacrificing quality on a continuous basis by consuming inadequate diet and eating less preferred food. They started reducing the quality of food intake by decreasing meal sizes and by only eating once or twice in a day in the past 30 days. The findings of food security status of the rice farmers in the Savanna and the Rainforest agro-ecological zones also revealed that 37.8% and 30.4% respectively were severely food insecure. Thus, the findings reveal that respondents in Savanna agro-ecological zone were more severely food insecure when compared with Rainforest agro-ecological zone. The finding for the pooled sample shows that about 33.3% of the rice farmers in Southwest were severely food insecure. This implies that about 33.3% households experienced high incidences of food insecurity. The condition of reducing meal sizes and the number of meals worsened each day. The three most severe conditions of going a whole day without eating, going to bed hungry and running out of food in the past 30 days occurred 'often' in the study area.

A possible explanation that can be used to explain observed differences in food security status in the two selected zones has to do with variation in temperature in these two agro-ecological zones. The scale of temperature variation in the Savannah zone is more severe than that in the Rainforest agro-ecological zone. Additionally, temperature increases in the Savannah agro-ecological zone can impede soils from being productive through increased levels of nitrate leaching and the lack of nitrates in the soil because of the heightened turnover rate of soil organic matter, which is a building block for soil fertility, sustainability and productivity in food production (Olesen and Bindi, 2002). Continuous temperature increases, coupled with limited rainfall, produce drier soil conditions through the high evaporation rates, resulting in the risk of wind erosion that undermines the topsoil and increases the possibility of salinity (Yeo, 1998). This resultant condition can jeopardize the production of food items in the Savannah agro-ecological zone due to their rooting mainly anchored in the topsoil layer. Consequently, increasing temperatures in Savannah agro-ecological zone could intensify respiration processes, accelerate development and hasten maturation without the plant completing proper growth processes, thereby reducing food production (Rötter and Van de Geijn, 1999; Olesen and Bindi, 2002).

Results of Linear Regression Model

The results of linear regression model were presented in Table 7 and it shows that there is significant relationship between socio-economic characteristics and food insecurity status of the respondents. The coefficients of respondents age in the Savanna and Rainforest agro-ecological zones (SRAEZs) were significant ($P<0.05$) and had a negative relationship with food security. This implied that increasing age of the respondents is associated with a decreasing probability of being food secure. This further implied that rice farmers are less likely to be food secure as they advance in age. As the rice farmers grows older, the energy and vigor to engage in rigorous farm activities reduces, leading to lower income and making them prone to food insecurity (Obayelu et al., 2021).The coefficient of the gender of the respondents was positive and significant at $P<0.01$ in the Rainforest agro-ecological zone. This shows that respondents who are male in the study area had higher probability of being food secure. This was in line with several other studies such as Oluyole et al. (2009), Omonona and Agoi (2007) used a household-based survey, COC and logit model to both classify cocoa farming and urban households to food security status and factor influencing them respectively.

Also, the coefficients of years of educational status in the Savanna and Rainforest agro-ecological zones were significant ($P<0.05$) and had a positive relationship with food security. This implied that increasing years of educational level is associated with an increasing probability of being food secure. Several previous studies also showed that the educational level of the household head is negatively related to household food insecurity (Amaza et al., 2009; Bashir et al., 2017; Gezimu Gebre, 2012; Idris & Gwary, 2008; Mango et al., 2014). The coefficient of household size in the Savanna agro-ecological zone was significant ($P<0.05$) and had a negative relationship with food security. This implied that decreasing household size is associated with an increasing probability of being food secure. Thus, households with a fixed income must distribute the available food among household members. Moreover, managing the food supply for all members of a household becomes more difficult when an additional member is introduced into the family while its income remains fixed. Jacobs (2009) found that larger households consume more food and thus need to increase their food expenditure and compete for scarce resources (Ndobo & Sekhampu, 2013), which makes them more likely to be food insecure compared to smaller or more nuclear households (Babatunde et al., 2007). The coefficient of farm size in the Rainforest agro-ecological zone was significant ($P<0.05$) and had a positive relationship with food security. This implied that increasing farm size is associated with an increasing probability of being food secure. Rahman and Islam (2013) showed that a positive relationship exists between households' food intake and farm size. Mannaf and Uddin (2012) also showed that large-farm owners are more likely to be food secure than small farm owners. This implies that large-farm owners are able to consume more food. The coefficient of rice farming experience in the Rainforest agro-ecological zone was significant ($P<0.05$) and had a positive relationship with food security. This implied that increasing rice farming experience is associated with an increasing probability of being food secure. Similarly, these findings indicated that as the rice farmers have more farming experience, as reflected in the increase in the number of years engaging in rice farming, the more likely the rice farmers become food secure. The coefficients of land tenure system in the Savanna and Rainforest agro-ecological zones were significant ($P<0.05$) and had a

positive relationship with food security. This implied that increasing ownership of rice farm land in the study area is associated with an increasing probability of being food secure. This result is also in line with Pankomera et al. (2009) and Bamire (2010 who observed that increase in the land holdings size of farm households in the dry Savannas of Nigeria improves probability of a household being food secures by 0.07 units. The F-ratio which determines the overall significance of the regression model is statistically significant at the 1% level in the Savanna and Rainforest agro-ecological zones. It therefore revealed that the independent variables significantly affect food insecurity. Also, socio-economic characteristics were not independent factors. Sixteen of the coefficients of Pearson correlation were significantly correlated, indicating Socio-economic characteristics are often, though not always, implemented in combination (Table 8).

Table 7. Results of Linear regression Model

	Savanna agro-ecological zone			Rainforest agro-ecological zone		
Variables	Coefficients	Standard error	P>t	Coefficients	Standard error	P>t
Age	-0.0376***	0.0147	0.00	-0.1036***	0.0114	0.00
Sex	-0.5299**	0.2215	0.03	0.1814***	0.0468	0.79
Marital status	-0.2186	0.3254	0.50	0.0471	0.2743	0.86
Years in school	0.0358**	0.0177	0.04	0.0140	0.0176	0.42
Farm size	0.0297	0.0308	0.33	0.2130**	0.1022	0.02
Household size	-0.0561**	0.0247	0.02	-0.0503	0.0564	0.37
Extension service	0.3387	0.2682	0.20	0.3255	0.2214	0.14
Farming experience	-0.0238**	0.0123	0.05	0.0023	0.0137	0.86
Rice farming experience	0.0133	0.0167	0.42	0.0603**	0.0239	0.01
Tenure system	0.2262**	0.0904	0.01	0.3786**	0.1664	0.02
Income	5.53e-08	1.86e-06	0.97	4.07e-06	3.66e-06	0.26
Constant	2.5808**	0.7874	0.00	0.8756	0.8135	0.283
No. of observation	225			352		
Adjusted R^2	0.2950			0.3722		
F ratio	19.181			16.903		

Source: Computed from field data, 2021.

Table 8. Correlation coefficients for linear regression equations

	S	MS	YS	FS	HS	E	FE	RE
S	0.041(0.32)							
MS	0.257**(0.00)	0.027(0.52)						
YS	-0.005 (0.90)	0.008(0.86)	-0.022(0.59)					
FS	0.031(0.46)	-0.034(0.41)	0.033(0.45)	0.132**(0.00)				
HS	0.134**(0.00)	-0.055(0.00)	0.058(0.16)	0.126**(0.00	0.255**(0.00)			
E	0.137**(0.00)	0.077(0.06)	-0.004(0.93)	-0.068(0.10)	0.028 (0.49)	-0.317**(0.00)		
FE	0.524**(0.00)	-0.023(0.58)	0.067(0.11)	-0.063(0.13)	0.107*(0.01)	0.200**(0.00)	0.063(0.13)	
RE	0.480**(0.00)	-0.054(0.19)	0.0048(0.24)	0.117**(0.00)	0.256**(0.00)	0.219**(0.00)	0.130**(0.00)	0.786**(0.00)

S: Sex; MS: Marital status; YS: Years in school; FS: Farm size; HS: Household size; E: Extension; FE: Farm experience; RE: Rice experience

Table 9. Analysis of T-Test of Food Security Status among Rice Farmers in the Savanna and Rainforest Agro-ecological Zones

Variables	Obs.	Mean	Difference	Standard deviation	t-value
Food security status					
Savanna zone	252	4.01	0.39***	4.96	2.94
Rainforest zone	352	5.16		4.67	

The Coping Strategies Respondents Adopt to Combat Food Insecurity

As shown in Figure 2, the coping strategy that is mostly adopted by the rice farmers in the study area is spending the whole day without food; this implies respondents skipping a whole day without eating as a result of food insecurity. This is followed by eating once per day, which implies to cut down the numbers of times food items consumed. So, as to cope with their shortage in food, the respondents tend to reduce the number of times food items they consumed per day. The next strategy is eating but not satisfied, it implies some of these respondents are just eating what is available not want they desire to eat in term of quality and quantity as a result of lack of food items. Also, borrow from friends and relatives is another strategy adopted by the respondents to cope with food insecurity. The least adopted coping strategies include spend on savings for other food project; send their children to look for food somewhere else; reduce the quantity of food consumed and change the type of food they eat and go for the less quality food items. This finding shows that majority of the respondents adopted different coping strategies to mitigate against food insecurity. This is similar to the study of Babatunde et al. (2018) who reported that various coping strategies were adopted by the farmers to cut down on the numbers of food items consumed.

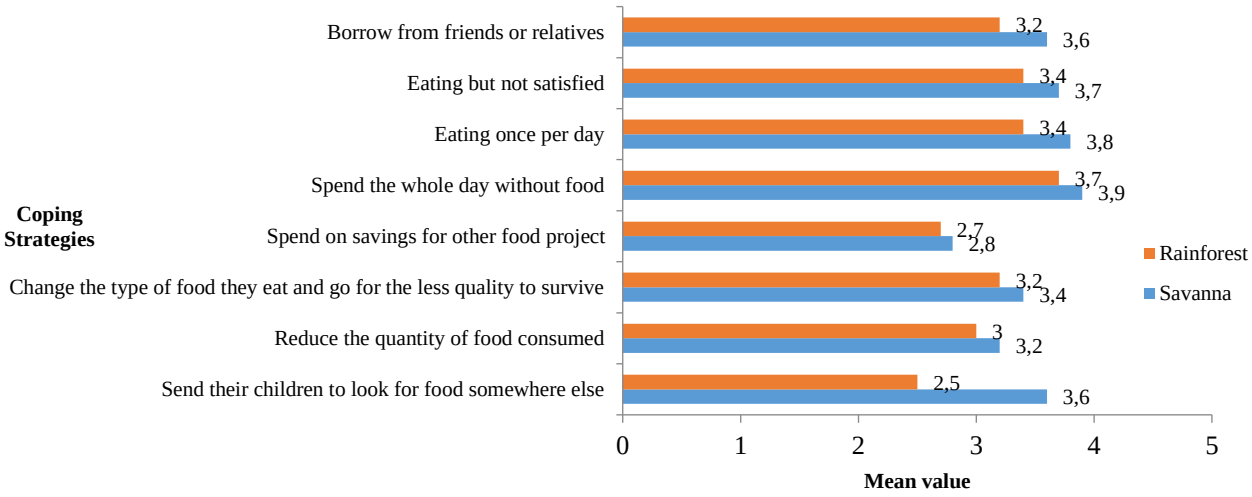

Figure 2. The coping strategies respondents adopted to combat food insecurity

Conclusion

The study assessed the comparative analysis of household food insecurity status among rice farmers in Savanna and the Rainforest agro-ecological zones in Southwest States, Nigeria. Majority of the rice farmers in the SRAEZs have household size of 5-8 persons, married, have good farming experiences, have small farm size and educated. The respondents in the SRAEZs were mildly food insecure and moderately food insecure respectively. Only 39.1% and 33.5% of respondents were classified as food secure, while others were food insure in the SRAEZs respectively. The major coping strategies adopted by the respondents against food insecurity include reduce the quantity of food consumed and eating but not satisfied. The socio-economic characteristics that drives food insecurity includes age, sex, years in school, farm size, household size, farming experience, rice farming experience and tenure system. It therefore recommended that age, sex, years in school, farm size, household size, farming experience, rice farming experience and tenure system are important drivers of household food security status that have to be taken into consideration by governments and development agencies wishing to promote the food security status of households in the study area.

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Effects of Land uses on Soils Quality in Rwandan Central Plateau Agro-Ecological Zone

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ABSTRACT

Conversion of land use from forest to agricultural uses modifies soil quality through physiochemical soil properties changes. This study was conducted in Rwanda's central plateau agro-ecological zone to evaluate the effect of forest and agricultural land uses on soil quality. The study was conducted in 2020. Soil samples were collected at the top, middle and bottom positions of each of the two land uses. We analyzed soil bulk density, soil moisture content, soil pH, soil organic matter (SOM), total nitrogen (TN), available phosphorus (Av P), and CEC for each position of the land uses. Data were analyzed using ANOVA in GENSTAT version 13. The results revealed that soil properties were significantly affected by land use change. Analysis of variances (LSD<0.05) results showed, however, that treatments were not significantly different within the same land use. The results showed that treatments from top position of forest lands had the highest mean values for soil organic matter and total N parameters with the respective mean values of 6.58 %, and 0.37 %. Treatments from middle position of forest lands had the highest mean values for soil moisture content and Av P parameters respectively with 23.60 % and 29.56 ppm. But, soil bulk density was high on top position of agricultural land with a mean value of 1.49 g/cm^3. Land users are advised to apply crop and soil management techniques which maintain soil quality and productivity on agricultural lands.

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Introduction

The land uses determine the fate of the soil quality (Tsadila et al., 2012). Soil quality can be defined as "the capacity of a specific kind of soil to function within natural or managed ecosystem boundaries to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation (Karlen et al., 1997). Intensive agriculture, deforestation and overgrazing significantly affect soil quality in different parts of world (Gupta, 2019; Tsadila et al., 2012).It is estimated that more than two billion hectares approximately 52 percent of worldwide agricultural lands is moderately or severely degraded which affect the livelihood of 1.5 billion people (FAO, 2018). Land use conversion into agricultural production generates adverse effects on soil quality (Yansui et al., 2004)

Land use conversion and poor agricultural practices are root causes of soil quality degradation in Rwanda. These result into soil erosion problems and soil fertility decline which affect land use potential (Bizimana, 2018). Deforestation and grazing land conversion to agriculture exposes soil to erosion which removes top soils along with nutrients and organic matter leaving behind unproductive soils. This is exacerbated by other factors such as heavy rainfall, inherent fragile soils, cropping on marginal steep slopes, continuous land tillage, and lack of sufficient soil and water conservation measures (GoR, 2018; Karamage et al., 2016). It was reported that on average 48t/ha/year and 39.2 t/ha/year of soils were lost through soil erosion in 2000 and 2015, respectively, translating into approximatively 110 and 89 millions of tons lost per year for the entire country (Nambajimana, et al., 2020). According to Byizigiro et al. (2020) soil loss was estimated to be at 38.4t/ha/year. Soil erosion reduces soil depth and increases fertility losses which affect soil quality and crop productivity. Soil particles, soil organic matter and nutrients are lost as results of erosion which cost around

US$ 34 million or at least 2 percent of the country GDP (REMA, 2009).

Soil quality degradation is also caused by nutrient and organic matter removal through continuous cultivation of arable lands (Andriesse & Giller, 2017). Continuous cultivation of lands with high nutrient demanding crops accompanied by agricultural practices which do not replenish the same amount of depleted nutrients is further degrading soil quality and impact crop productivity (Okalebo, 2009). Organic inputs such as crop residue and manure applied to improve soil quality were reported to be of low quality and insufficient in tropical region (Palm et al; 2001). A survey by NISR (2018) in a certain study showed that nearly half of all cultivated plots received organic fertilizers and a quarter of all plots were applied with mineral fertilizer for each agricultural season in year 2018. As consequence, soils were reported to have high acidity, low organic matter, and poor soil nutrient contents mostly in Congo-Nile watershed divide and central plateau agro-ecological zones of Rwanda (Muhinda et al.,2009). These soils have high soil acidity, nutrient deficiencies such as N, P, Ca, Mg and high Al toxicity (Nzeyimana et al., 2013; Mukuralinda et al., 2010). Soil quality degradation affects agricultural sector which is still a pillar of Rwandan economy. The sector contributes up to 29 percent of GDP, occupies 70 percent of the labor force (NISR, 2019) and generates more than 45 percent of the country's export revenues (NISR, 2015).

Contrary to agricultural land use, forest land use with permanent cover improves soil quality through deep and extended rooting systems that hold soil particles together preventing them from detachment and soil erosion initiation. Soil erosion severely affects deforested areas which loads waterbodies with siltation sediments (Zhao et al., 2009). Forest canopy intercepts rain drops and weakens its impacts on soils and slows down soil erosion. With forests cover soil erosion under forest lands was reported to be low. This is mirrored in Rwandan forest cover which accounted for 21% of the total land but contributes only 0.2% of total soil erosion (Karamage et al., 2016). As results, soil water storage increases through increased infiltration rate and improved soil aggregate stability (Sharma and Sharma, 2004). Forests root exudates and litter falls which accumulate on soils improve soil organic matter and nutrients which further ameliorate forest soil quality.

Land uses are affected differently. It varies between regions, countries and even at zone level. Rwanda Central plateau agricultural zone is the region dominated by agriculture land use with small patches of forestlands. The big part of this agricultural zone was brought under cultivation since many years ago. Although many studies were carried out in the area on nutrient contents of agricultural lands, scanty information exist on how land use affects soil quality. The objective of the study was to analyze the long-term effect of land use on soil quality in central plateau agricultural zone of Rwanda.

Materials and Methods

Site Description

The study was conducted in Ruhande Arboretum and nearby agricultural farms both located in Huye District, in the Southern Province of Rwanda. The District is located in the central plateau agro-ecological zone with average altitude of 1700 m above sea level. It enjoys sub-tropical climate with average rainfall of 1160 mm and average temperature of 20^{o}C (Huye Ditrict, 2018; Kalinganire & Hall, 1993). The District has only about 10 percent of forests as the remaining percent was converted into agricultural lands. Note that 85 percent of the population practice farming activities (Huye District, 2013). The study area has two cropping seasons. The first season starts in September and ends in January and the second from February to June. Ruhande Arboretum is a forest plantation of 200 hectares with 529 plots. It was established by colonial rulers in 1934 (Mugunga, 2009).

Methods

Sampling procedure started in agricultural season B (February- June) of 2020 by removing all dead plants at each sampling point. The experimental research was randomized complete block design with two land use types which were (1) forestland and (2) cultivated land. For each land use, soil samples were collected at three levels (top, middle and at the base of the hill) and replicated three times. In total, 18 samples were collected. Sampling depth range was 0-20 cm. soil samples from the corner and center of each plot were mixed to generate composite sample and was done following transect sample. The plot size was 4×5 m. undisturbed soil samples were also collected on the same plots and depths. Hand soil auger and steel core cylinder were used to collect disturbed and undisturbed soils respectively. All collected samples were brought to the soil and plant analysis laboratory of University of Rwanda located at Huye campus for analysis.

Laboratory Analysis

After data collection, we brought samples to the laboratory for analysis. Gravimetric method was used to analyze both soil moisture content and bulk density. For disturbed soil samples: soil texture was analyzed using Densimetric method of Bouyoucous; sensitive glass electrode method was used for Soil $pH_{(water)}$; soil organic matter content (SOM), available phosphorus (Av P) and total nitrogen (TN) were measured by UV-Visible Colorimetric method. Kjeldhal distillation method was used to analyze cation exchange capacity (CEC). All analysis followed the protocol developed by Okalebo et al.(2002).

Data Analysis

Data ware analyzed using analysis of variance (ANOVA) which tested the difference in soil parameters analyzed. To determine the significant difference between treatments, we used comparison between two treatments mean and least significant difference (LSD) at 5 %. We considered the results to be statistically significant once the difference between two treatment means was greater than least significant difference (LSD). Data analyses were processed by GENSTAT version 13.

Results

Results for soil bulk density and soil moisture content are presented in Table 1.

Table 1. Mean value of some soil properties in relation to land use (20 cm of depth) and position

Land use type	Position	Parameters						
		Soil Density	Moisture content	pH H_2O	SOM	Total N	Av. P	CEC.
		g/cm^3	%		%	%	ppm	$Cmol_{(+)}/kg$
Forest	Top	1.29c	22.53a a	5.5a	6.58b	0.37a	28.56a	16.96a
	Middle	1.32bc	23.60a a	5.5a	6.54b	0.32a	29.56a	16.26a
	Bottom	1.36abc	23.31a a	5.4a	6.16ab	0.35a	29.22aa	17.94a
Agriculture	Top	1.49a	18.45b b	5,0b	5.00ab	0.19b	21.22b	10.86b
	Middle	1.47ab	17.38b b	5,0b	4.63a	0.23b	20.56b	11.56b
	Bottom	1.43abc	17.67b b	4.9 b	4.60a	0.19b	20.22b	9.88b
F-P (5 %)		0.06	<.001	0.020	0.04	<.001	0.001	0.006
LSD		1.29	2.36	0.46	1.54	0.06	4.37	4.08
CV %		5.5	6.1	4.8	14.7		9.3	15.6

Discussion of Results

Soil Bulk Density (BD) and Soil Moisture Content (MC)

Results for soil bulk density and soil moisture content are presented in Table 1. The results showed significant difference between treatments for both soil bulk density (BD) and soil moisture content (MC). The lowest BD value ($1.29g/cm^3$) was observed on the top position of forest land and the highest value ($1.49g/cm^3$) was found on the top of agricultural land. For MC, the highest value (23.60 %) was found in the middle of forest land and the lowest value (17.38 %) was recorded in the middle of agricultural land. The small result of BD in forest land could be ascribed to its high organic matter content as compared to agricultural land. The results concur with that of Gol (2009) and Hajabbasi et al. (1997) who reported low BD in forest lands compared to agricultural lands. It was reported by Weil & Brady (2017) that intensive tillage reduces soil organic matter and breaks soil structure which increases soil bulk density

The higher values of moisture content in forest areas could be assigned to its high organic deposition compared to agricultural land. The results are in line with that of Manpoong and Tripathi (2019) who reported high moisture content in forest lands and attributed it to high organic matter content and plant communities which maintain moisture content in natural forests compared to other types of land uses. Fesha et al. (2002) reported high soil water retention in non-cultivated treatments as compared to conventional treatments.

Soil pH (H_2O) and Soil Organic Matter (SOM)

Analysis of variance showed the significant difference ($P<0.05$) among treatments for pH. The highest pH value was observed on top and middle positions of forest land with 5.5. Small pH value was obtained on bottom position of agricultural land. No significant difference in mean values of pH observed within the same land use. The small pH value in agricultural lands could be a result of nutrients loss through crop harvests and soil erosion which carry them and not being replenished. The results concur with those reported by Emiru & Gebrekid (2013) who attributed low pH in agricultural lands compared to forest land to leaching of cation bases from surface layers, accelerating soil erosion which eventually drains them into streams and their removal through crop harvests. Fetene & Amera (2018) ascribed low pH values on grazing lands and agricultural lands to soil disturbance which cause soil erosion and deplete basic cations. He also attributed it to base cation losses through leaching and use of diammonium phosphate ($(NH_4)_2HPO_4$) which release H^+ ions that replace them in soil solution.

For SOM, the highest value (6.58 %) was obtained on the top position of forest land while the bottom position of agricultural land use had the lowest value (4.60 %). Forest land use had higher SOM than agricultural land uses. This could be attributed to the permanent forest cover which improves SOM through accumulation of plant litter and roots exudation on upper layers of soils and their low rate of decomposition. Contrary to forest land, agricultural land uses experience continuous nutrient removal trough harvests and leaching, rapid decomposition of SOM and its removal through soil erosion. These results concur to those of Moges et al. (2013) and Selassie et al. (2015) who reported that farming lands had significantly lowered SOM contents compared to protected forests. Low SOM content was attributed to low quantity of SOM returned in the farms, its high oxidation that takes place as well as its loss through soil erosion. According to Wasige (2014) land cover type was the main cause of low SOC content in agricultural land compared to forest land. Liu at al. (2006) and Hajabbasi et al. (1997) reported that continuous cultivation exposes SOM to decomposition which decreases its content in soils.

Total N, Available P and CEC

Analysis of variance showed significant difference among mean values of different treatments for total N, available P and CEC parameters. Total N, available P and CEC were significantly higher on the top, middle and bottom of forest land use while all levels of agricultural land use showed the lowest values. The highest values for total N, available P and CEC were recorded, respectively; on top (0.37 %), middle (29.56 ppm) and on bottom with (17.94 $cmol_{(+)}/kg$). The lowest values were observed for total N, available P and CEC, respectively; on the top (0.19 %), on the bottom (20.22 ppm), and on the bottom ($9.88cmol_{(+)}/kg$). However, mean values were not statistically different within each land use. Low TN and Av. P contents in agricultural lands could be ascribed to low fertilizer use both organic and mineral, nutrient removal through harvests, and nutrient losses through soil erosion and leaching as compared to forest lands. The results are in line with those of Wang et al (2001) and

Moges et al (2013) who reported lower TN content and attributed it to nutrients removal through crops harvests and inadequate application of fertilizers in farmland. Solomon (2002) reported low TN content in grazing and farming lands which is attributed to mineralization of SOM and leaching of nitrate-N. According to Selassie et al (2015) TN content was high in forest land in comparison to agricultural land due to N which is bound in organic carbon. Wang et al. (2001) reported high TN content in uncultivated land use as compared to cultivated land and attributed it to destructive soil management practices which cause soil erosion in cultivated lands.

The results of Av.P were higher in forest land than in agricultural land. According to Fetene and Amera (2018), who reported similar results, Av.P followed the trend of SOM of which it is associated with and was affected by conversion of natural forest into cultivated land. Emiru & Gebrekid (2013) reported high Av. P in forest lands. Conversely, agricultural lands had lower Av. P in forest land. Mukuralinda *at al*.(2010) reported low quantity of available P in agricultural land in southern Rwanda caused by native soils with poor P content, its retention by aluminum and iron oxides and insufficient use of fertilizer. Abera and Wana (2023) reported that agricultural land uses without land management practices also showed lower Av P as compared to that one with land management practices.

The results showed that CEC was higher in all positions of forest lands compared to that of agricultural lands. The trend of CEC variation among land uses nearly followed that of SOM. High CEC content in forest land could be attributed to the limited disturbance on soil structure and accumulation of SOM from tree biomass. Normally, CEC depends on the amount and types of clay and SOM contents which are negatively charged. Tesfahunegn and Gebru (2020) and Mandal et al. (2013) reported that CEC was higher in forest land and was related to its high OM and clay content. Emiru and Gebrekid (2013) attributed low CEC content in agricultural land to low quantity of SOM content.

Conclusion and Recommendations

The findings of this study revealed that agricultural land uses reduce the soil quality than forest land uses. The analysis of variances have shown that there was statistical difference (lsd<0.005) between treatments from forest lands and agricultural lands but there was no statistical differences among treatments within the same land use. Treatments under forest land uses had shown high mean values on soil moisture content, soil pH, and soil organic matter, soil N, Av P, and CEC. Treatments of top position of forests had the highest mean values for soil organic matter and TN with 6.58 % and 3.7 % respectively, while mean values were high on middle positon of forest land uses for soil moisture content and Av P parameters with 23.60 % and 29.56 ppm as their respective mean values.

The highest bulk density was obtained on the top position of agricultural land with 1.49 as mean value. Soil disturbances through some agricultural practices had contributed to speed up soil properties dynamics in unsustainable manner. This study advice to use agricultural practices that minimize soil disturbance and reduce soil erosion by maintaining land cover such as perennial crops, agroforestry, and soil and water conservation measures. Application of organic fertilizers such as crop residues, manure and compost combined with mineral fertilizer can contribute to reduce nutrient deficits, improve soil quality and sustain crop productivity.

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Production of Tomato Seedlings Submitted to Treatments with Foliar Application of Paclobutrazol

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A B S T R A C T

Paclobutrazol (PBZ) is a growth regulator that widely used in horticulture and in the tomato seedling growth to compact the shoots, increase the stem diameter and, root biomass, allowing more tolerance of the seedlings against adverse weather conditions. The objective of this work was to evaluate the rates of paclobutrazol (0, 4, 7, 10 and 13 mg L^{-1}) applied 15 days after sowing by foliar spray on the growth, chemical composition and xylem vessel number of tomato seedlings cultivated in two periods. The PBZ regardless of the application rate reduced the height of tomato seedlings in both growth periods. The basal stem diameter and leaf area were increased with 13 mg L^{-1} of PBZ. The lignin percentage also increased with 10 and 13 mg L^{-1} of PBZ as compared to control for both periods. The number of xylem vessel was not affected by PBZ application on the seedlings in the first period. PBZ application at rates of 7 and 10 mg L^{-1} increased the xylem vessel number in the second period. In general, the application of 13 mg L^{-1} of PBZ generated seedling more robust to overcome climate adversities. These findings contribute to science by providing insights into how much dosage of Paclobutrazol can be utilized to modify plant morphology and enhance seedling resilience, offering potential applications in agriculture for improving crop yield and sustainability, particularly in challenging environmental conditions.

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Introduction

Tomato (*Solanum lycopersicum* L.) is among the most important vegetable produced in the world, which production in 2018 was approximately 182 million of tons in an area of 47.6 million of ha (FAO, 2020). The tomato's high productivity depends on the seedling quality, among other factors characterized by compact shoots and vigorous root systems that allow more tolerance against adverse weather conditions as high temperatures and excessive rainfalls (Oliveira et al., 2022). Seedling growth is controlled for genetic, environmental conditions, and applied agricultural practices.

Agricultural practices such as growth regulator applications have been used to compact the plants and increase stem diameter (Desta and Amare, 2021). They are synthetic compounds that reduce the stem elongation by decrease of cell elongation and division rate.Among growth regulators, paclobutrazol (PBZ) [(2RS, 3RS)-1-(4-chlorophenyl)-4,4- dimethyl-2-(1,2,4-triazol-1-yl) pentan-3-ol] is a compound of the chemical group of triazoles that acts as an inhibitor of gibberellic acid biosynthesis by suppressing the oxidation of ent-kaurene to ent-kaurenoic acid through deactivating cytochrome P450-dependent oxygenase (Graebe, 1987). The inhibition of gibberellins biosynthesis causes alterations in plant hormonal balance and promotes prominent reduction of stem elongation (Rademacher, 2000). The application of PBZ is responsible to compact seedlings without damaging the leaf area and to allow an increase of approximately 37% in the dry matter of roots (Seleguini et al., 2013). Then In addition to this, PBZ has been used to reduce the height and increase the stem thickness of tomato seedling in the nurseries. PBZ might enhance the chloroplast differentiation and

chlorophyll biosynthesis and prevents chlorophyll degradation attributed to the increase in endogenous cytokinin content (Banon et al., 2002).

Paclobutrazol also can be related with increase of compound amounts such as lignin, hemicellulose and cellulose, and xylem vessel number of the plants, resulting in high seedling quality (Chen et al. 2011). The chemical composition of stem has an impact on it is the mechanical strength and rigidity. Lignin is an integral structural carbohydrate of secondary cell walls, giving mechanical strength to the plants (Chen et al. 2011). In addition, triazoles as PBZ can induce anatomical modifications mediated by changing the hormonal balance of plant. In potato crop, PBZ increased root diameter by increasing the width of cortex and by enhancing the formation of more secondary xylem vessels (Tsegaw et al., 2005).

This study aimed to obtain ideal tomato seedlings, seedlings with more compact and thicker stems perform better during tomato development and to evaluate the effects of paclobutrazol application on the morphology, anatomy and development of tomato seedlings.

Materials and Methods

Experimental Site

The experiment was carried out in a commercial nursery located in the city of Mogi Guaçu (SP), Brazil, located at 22º22' S, 46º56' W at 617 m altitude in the greenhouse (10 x 25 m of height and wide), covered with diffusor plastic. The water or nutrient solution was applied with a mobile irrigation bar.

Tomato Seedlings and Treatments

Tomato cultivar of Serato F1 hybrid was used as it is well known as persimmon tomato is very vigorous, precocious and produces large and heavy fruits (average of 250 g), and it has resistance to nematodes and TSWV (headworm).

The tomato seeds were sown in polyethylene trays of 128 seeds filled with a substrate composed by coconut fiber. The soil substrate characteristics were as N = 0.76%, P_2O_5 = 0.34%, K_2O = 1.22%, Ca = 0.69%, Mg = 0.25%, S = 0.41%, organic matter = 86.00% and carbon = 47.80% in dry matter; Na = 500 mg kg^{-1}, Cu = 100 mg kg^{-1}, Fe = 2600 mg kg^{-1}, Mn = 266 mg kg^{-1}, Zn = 148 mg kg^{-1}, C / N = 63/1 and pH = 4.50.

The experimental design was completely randomized with five rates of paclobutrazol (0, 4, 7, 10 and 13 mg L^{-1}) and four replications. The tomato seedlings were sprayed with paclobutrazol at 15 days after sowing (DAS).

Evaluated Characteristics

At 35 DAS, the following characteristics were determined: plant height, leaf area, upper stem diameter of the seedlings, lower stem diameter of the seedlings, dry weight of the shoot, root dry weight, root length, surface diameter of roots and branches, xylem vessel number and cellulose, hemicellulose and lignin contents of the stems.

Growth Parameters

Plant height was measured from the base of the hypocotyl to the apex of the seedling with a digital caliper. The top and medium diameters of the stem were measured with a digital caliper. The shoots and roots of five seedlings per replication were dried at 65°C for 48 hours. The dry matter of each part was weighed on a digital scale. The leaf area was obtained trough of Image J software, and it was expressed in cm^2.

Five root systems of each replication were submerged in containers with distilled water for 30 minutes. This procedure facilities the root washing process to remove substrate particles. For the morphological analysis, a WinRHIZO Pro 2007a system (Régent Instr. Inc.), coupled to an Epson XL 10000 professional scanner equipped with additional light unit (TPU) was used. A definition of 400 (dpi) was used for measurements of root morphology, as described by Flores et al., (2018) and Jabir et al., (2017). The roots were laid in an acrylic vat 20 cm wide by 30 cm long containing water. The use of this accessory allowed the capture of images in three dimensions, also avoiding the overlapping of the roots. The readings were taken individually to avoid image overlap. The evaluated characteristics were root length, root system density and root ramifications.

Xylem Vessel Count

Portions of the tomato stem were fixed in a Karnovsky system (Karnovsky, 1965), and sectioned in a rotating 40 micrometer microtome. Sections were clarified with sodium hypochlorite and double stained with Astra Blue (0.5%) and Safranin (0.5%)and mounted on a slide with 50% glycerin. The images were captured on a Leica DM LB trinocular microscope coupled to the Leica DC 300 F camcorder and xylem vessel counts were performed using Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD, USA) (Figure 1).

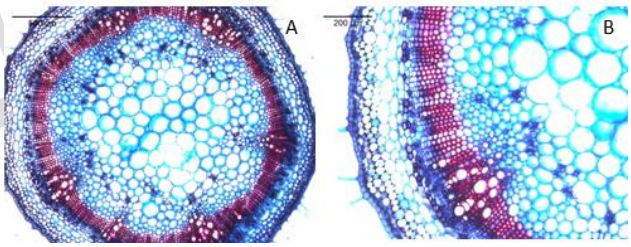

Figure 1. Overview of a cross section of fresh tomato seedling stem (A), and the xylem vessels measured in the analysis (B)

Cellulose, Hemicellulose and Lignin Concentration

The samples of 5 g of the stem were placed on a sachet prepared with filter paper. The solvent used for extraction was 95% ethanol. The procedure was performed on a Soxhlet apparatus for 6 hours under reflux. After the extraction, the samples were withdrawn and the cooling time of the samples was maintained at room temperature. Then, 300 mg were placed in crucible and dried until constant dry weight to calculate the moisture content.

The samples of 300 mg of the extracted stem were weighed into the test tubes, to which 3 mL of 72% (v/v) sulfuric acid (d = 1.84) were added and placed in a water bath at 30°C, submitted to shaking at 75 rpm for one hour. The material was diluted to a concentration of 3% (v/v) acid by the addition of 79 mL of distilled water and heated at 121 °C for an additional hour.

Soluble and insoluble lignin: The resulting hydrolysate material was vacuum filtered and placed on a porous crucible and dried to a constant mass value at 105 °C. The determination of the insoluble lignin was made by gravimetry and the soluble lignin at 205 nm by UV-visible Shimadzu Mini1240.

The liquid fraction from the hydrolysate was used for the determination of carbohydrates and organic acids by high performance liquid chromatography (HPLC), which vials with this liquid fraction were prepared. During the vial preparation the material was previously filtered through a 0.45 µm cellulose nitrate membrane (Sartorius Co.). Carbohydrates were analyzed on an Aminex HPX-87H column (300×0.8 mm) in a Shimadzu chromatograph LC-20AT. The mobile phase used was ultra-pure water (18 mΩ) at 0.6 mL min^{-1} flow rate and 45 °C. ELSD Shimadzu LT-11 at 80 °C, 350 kPa pressure and gain equal 7 was used as detector. Acetic acid determination was performed on the HPX-87H column, with mobile phase of H_2SO_4 at 0.5 $mL.min^{-1}$ and 40 °C. The employed detector was DAD (Shimadzu SPD-M20A), operating at the wavelength of 210 nm. The conversion of fructose into 5-hydroxymethylfurfural (HMF) and furfural were analyzed through C-18 column (Shim-pack XR-ODS 3) and the mobile phase used was acetonitrile/acidic water with 1% acetic acid in the proportion 1:8. The detector used was DAD (Shimadzu SPD-M20A) with flow of 0.8 mL min^{-1} and 25 °C. The wavelength for HMF analysis was 285 nm and for furfural was 275 nm.

Results and Discussion

In this research, the PBZ reduced the height of tomato seedlings in both growth periods. The applications of 10 and 13 mg L^{-1} of PBZ reduced, on average, 36.2% and 35.4% the seedling height as compared to control, in the first and second growth period, respectively (Table 1). The reduction of plant height by PBZ application on the shoots is related to inhibition of ent-kurene conversion to ent-kaurenoic acid, in the gibberellins biosynthesis, which results in reduced levels of gibberellic acids all types, causing a decrease in elongation rate and cell division (March et al., 2013; Desta and Amare, 2021; Syahputra et al., 2018). Velázquez et al. (2018) also observed that PBZ reduced 45% the size of tomato seedlings and found retarded growth of tomato plants with PBZ applied by foliar sprays.

In contrast to the height, basal stem part diameter (D1) increased 21.3% with application of PBZ at rate of 13 mg L^{-1} during the first period. In the second period, the applications of PBZ at rates of 10 and 13 mg L^{-1} increased 17.4% and 15.5% the D1, respectively (Table 1). Thus, the lower H/D1 ratio with PBZ than control allowed a balanced growth relationship between seedling height and diameter, promoting seedlings more robust.

The seedlings treated with PBZ had smaller leaf area than control, which rate of 13 mg L^{-1} reduced 35% this characteristic, for both growth periods. According to Tsegaw and Hammes (2005) and Desta and Amare (2021), triazoles as PBZ might cause morphological changes on the leaves as reduced leaf area and increased leaf thickness. This reduction of foliar area did not generate abnormal leaves in the seedlings treated with foliar sprays of PBZ (Tsegaw and Hammes, 2005).

The dry biomass of shoots (SDM) and roots (RDM) were not affected by PBZ foliar application. Thus, the decrease of seedling size and foliar area were not sufficient to enhance the biomass of roots. These results contradict the role of PBZ in reduction of the leaf expansion rate with reduction of the sink strength of the leaves and increase of the carbon flux to the roots as verified by Berova and Zlatev (2000). The authors found decrease in plant height and increase in root development of tomato seedlings with foliar application of 25 mg L^{-1} of PBZ.

The root quality did not increase with PBZ applied by foliar spray in the seedlings, for the first period. However, for second period, the length, area and forks number of roots increased with PBZ foliar spray at rate of 10 mg L^{-1} (Table 2).

Table 1. Height (H), basal stem part diameter (D1), upper stem part diameter (D2), leaf area (LA), shoot dry mass (SDM) and root dry mass (RDM) of tomato seedlings under rates of paclobutrazol (PBZ) during the growth periods. Means with the same letter within a column were not different by LSD test (P<0.05).

First growth period						
Rates (mg L^{-1})	H (cm)	D1 (cm)	D2 (cm)	LA (cm^2 pl^{-1})	SDM (g pl^{-1})	RDM (g pl^{-1})
0	19.719a	2.30b	2.24a	21.12a	0.73a	0.114a
4	15.465b	2.28b	2.34a	17.2b	0.59a	0.106a
7	13.500c	2.21b	2.23a	16.65c	0.64a	0.103a
10	12.810c	2.32b	2.33a	16.53c	0.67a	0.108a
13	12.350c	2.79a	2.35a	13.67d	0.58a	0.105a
LSD	1.95	0.25	0.25	3.50	0.17	0.02
Second growth period						
Rates (mg L^{-1})	H (cm)	D1 (cm)	D2 (cm)	LA (cm^2 pl^{-1})	SDM (g pl^{-1})	RDM (g pl^{-1})
0	20.77a	1.74c	1.90b	23.14a	1.350a	0.211a
4	15.22b	1.96ab	2.10a	18.88b	1.275a	0.228a
7	13.56c	1.87bc	1.97ab	18.25bc	1.33a	0.213a
10	13.83c	2.04a	2.10a	16.54c	1.37a	0.223a
13	13.01c	2.01ab	2.05ab	14.89d	1.30a	0.234a
LSD	1.04	0.14	0.15	3.25	0.29	0.07

Table 2. Root length (L, mm), root area (RA, cm^2) and forks number (FN) of tomato seedlings under rates of paclobutrazol (PBZ) during the growth periods. Means with the same letter within a column were not different by LSD test (P<0.05).

First growth period			
Rates (mg L^{-1})	L (mm)	RA (cm^2)	FN
0	222.12a	7.94a	1459.20a
4	262.61a	8.35a	1472.00a
7	250.44a	7.53a	1269.30a
10	246.08a	7.77a	1403.25a
13	231.90a	8.23a	1501.05a
LSD	56.54	1.76	356.97
Second growth period			
Rates (mg L^{-1})	L (mm)	RA (cm^2)	FN
0	68.58bc	2.64c	476.40bc
4	70.48bc	3.88b	614.15b
7	103.87b	6.24a	881.65a
10	154.96a	6.23a	969.30a
13	39.82c	2.07c	342.70c
LSD	27.97	0.83	235.21

Table 3. Amounts of hemicellulose, cellulose and lignin in the stem of tomato seedlings. Means with the same letter within a column were not different by LSD test (P<0.05).

First period			
Level (mg L^{-1})	%Cel	%Hem	%Lig
0	30.68e	17.72a	14.67c
4	43.38d	17.69b	09.33d
7	45.07bc	12.98e	07.56e
10	49.59b	15.30c	19.03b
13	51.93a	13.17d	29.69a
LSD	0.48	0.18	0.18
Second period			
Level (mg L^{-1})	%Cel	%Hem	%Lig
0	28.33d	15.69bc	14.03d
4	29.68c	14.27e	12.56e
7	35.64b	26.69a	15.70c
10	35.06b	14.52d	28.09b
13	44.14a	15.96b	33.34a
LSD	0.03	0.02	0.02

PBZ might generate an increase in cytokinin levels (Burondkar et al., 2016; Desta and Amare, 2021; Soumya et al., 2017) leading an intensification of cell division and, consequently an increase of area and forks number of roots.

The chemical composition of the seedlings was investigated in order to understand if there are structural changes resulting from the application of PBZ. For both crop cycles, the percentage of cellulose increased in the stems of the seedlings with increase of PBZ rates (Table 3). The lignin percentage also increased with 10 and 13 mg L^{-1} of PBZ as compared to control in the first period. For second period, similar results were obtained with 7, 10 and 13 mg L^{-1}. These results are in line with other researches in the literature (Ayvaci et al., 2023; Si et al., 2023).

The lignification process as well as the formation of other polymers are dynamic and are activated by the regulation of several genes that control this phenomenon as function of stress (Wang et al., 2013). According to Srivastava et al. (2017), abiotic stress as PBZ application in the seedlings can also induce lignification in the walls of cells that do not normally lignify under non-stress. Lignin is an integral structural carbohydrate of secondary cell walls, generating mechanical strength to plants (Chen et al. 2011). Therefore, an increase of lignin content results in rigidity of stem (Peng et al., 2014).

However, hemicellulose content decreased with PBZ rates applied by foliar spray in the first period. For second period, the PBZ rates of 4, 7 and 10 mg L^{-1} also reduced this compound content as compared to control. According to Xu et al. (2017), the increase of lignin and hemicellulose contents together increased the stem mechanical strength. Therefore, our results suggest that the lignin and cellulose accumulations are associated with lodging resistant of tomato seedlings.

Another variable analyzed was the count of xylem vessel number in order to verify if the compaction of tomato seedlings was associated by an increase of the vessel number of xylems. The number of xylem vessel was not affected by PBZ application on the seedlings in the first period (Figure 2). For the second period, the PBZ application at rate of 7 and 10 mg L^{-1} increased the xylem vessel number (Figure 3), corroborating with studies obtained by Tsegaw et al. (2005) in potato crop, where PBZ induced shorter and thicker stem associated with more secondary xylem vessels than control.

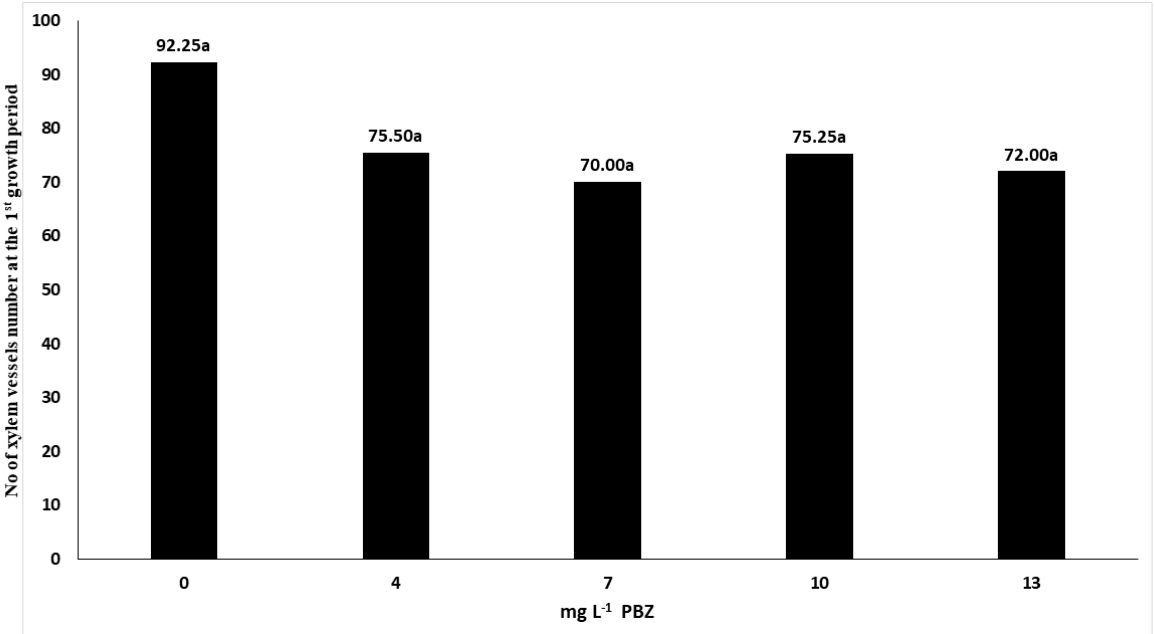

Figure 2. Xylem vessels number under rates of paclobutrazol (PBZ) during the first growth period.

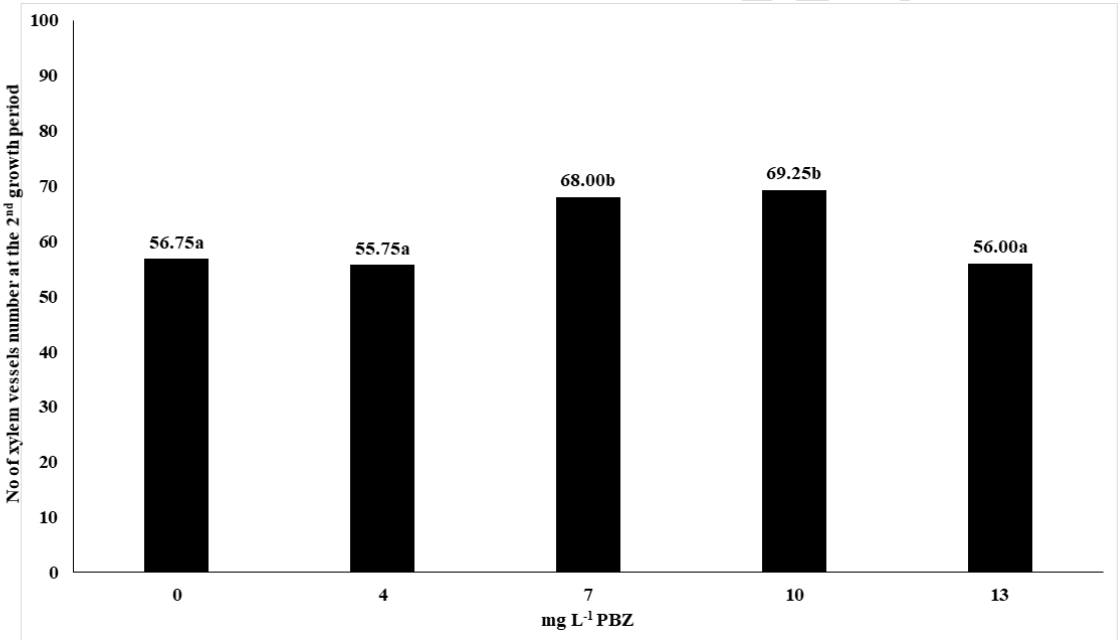

Figure 3. Xylem vessels number under rates of paclobutrazol (PBZ) during the second growth period.

In the study reported by Mohammadi et al. (2017), PBZ application at rate of 50 mg L^{-1} increased the xylem cell number and vessel cell size of juvenile plants of radish. In yellow passion seedlings, the higher stem diameter induced by PBZ also was related to a higher xylem vessels number (Teixeira et al., 2019).

In Chrysanthemum cultivars Jaguar Red, Snow White, and Fiji White, the utilization of PBZ at various concentrations caused in distinct influences on the plant anatomy (Lailaty and Nugroho, 2021). Notably, Jaguar Red exhibited greater stem thickness and stomatal density compared to Snow White and Fiji White (Lailaty and Nugroho, 2021). Moreover, the use of 150 ppm PBZ raised leaf thickness and stem diameter, while 100 ppm PBZ enhanced the size of guard cells and tissue dimensions (Lailaty and Nugroho, 2021). Ultimately, the optimal concentration for potted Chrysanthemum was determined to be 150 ppm (Lailaty and Nugroho, 2021).

Conclusions

Paclobutrazol modified the morphology of tomato seedlings. It induced anatomical changes such as reducing height and foliar area of seedlings and increasing diameter of stems. PBZ also improved the cellulose and lignin accumulation in the stems, thus increasing mechanical strength of seedlings. Therefore, these findings are valuable by using PBZ as a management practice for producing seedlings more robust to overcome the adverse weather conditions.

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Conflict of Interest Statement: The authors of the article declare that there is no conflict of interest.

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Silicon Improves Cold and Freezing Tolerance in Pea

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ABSTRACT

The most significant crop losses worldwide occur due to unfavorable temperatures such as heat, drought, cold, and freezing. Minerals like silicon can play important roles in the growth, development, and stress responses of plants. In this study, changes in stem/root length, dry weight, relative water content and silicon content, of peas under cold and freezing stress, as well as antioxidant system indicators such as proline, malondialdehyde, hydrogen peroxide, and chlorophyll levels, ion leakage, and the expressions of genes coding for the topoisomerase *TOP2* and DNA helicase *PDH47* enzymes, which play important roles in the replication, transcription, and repair of DNA molecules, were examined in root and stem tissues in the presence of two different concentrations of silicon. The results of the study showed that silicon application under cold and freezing stresses has induced various changes in pea metabolism, including increases in cell membrane integrity parameters and superoxide dismutase enzyme activity, as well as increase in the expressions of *TOP2* and *PDH47* genes. These changes have been found to have positive effects on the pea cold and freezing tolerance.

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Introduction

The abiotic stresses such as high and low temperatures and drought are major factors worldwide causing significant crop losses in agricultural plants (Jahed et al., 2023; Jan et al., 2023). Pea (*Pisum sativum* L.) cultivation involves seed planting in late winter or early spring, exposing plants to cold and freezing stresses. When these stresses coincide with drought during the seed formation period, it can lead to the loss of nearly the entire crop (Larran et al., 2023). Peas, a significant source of protein and carbohydrates, enrich the soil through the *Rhizobium* symbiosis specific to legumes, and due to their ability to fix nitrogen. They are annual leguminous plants that provide the highest yield per unit area under suitable weather conditions and play essential roles in sustainable agriculture worldwide (Stagnari et al., 2017). The most limiting factors for cultivation on larger expanses globally are the cold and drought stresses frequently encountered by the plant (Ding & Yang, 2022).

Although cold and freezing stresses fundamentally operate through similar mechanisms, they involve distinct components. Cold stress leads to various adverse effects, including a decrease in enzyme activities, stiffening of cell membranes, destabilization of protein complexes, stabilization of RNA secondary structures, accumulation of reactive oxygen species, retardation of photosynthesis, and leakage in the cell membrane. In advanced stages, cold stress can result in cell death (Manasa et al., 2022). The primary cause of freezing stress is the formation of ice within tissues. For instance, structures with very low water content, such as seeds, can maintain their vitality at low temperatures without showing signs of damage. Freezing stress primarily manifests its harmful effects through damage caused by advanced dehydration in cell and organelle membranes. The major genetic responses triggered during freezing stress involve an increase in the expression of *CBF* genes, which initiate transcriptional regulation, and mRNA degradation regulated by small RNAs through precursor mRNA cleavage, a post-transcriptional regulatory mechanism (Chinnusamy et al., 2007; Sunkar et al., 2007).

In the conducted studies, the biochemical and genetic responses of peas to cold and freezing stress have been particularly focused on the plant's photosynthetic efficiency and carbon or phosphate compound metabolism (Georgieva & Lichtenthaler, 1999; Streb et al., 2003; Stupnikova, 2006; Srivastava et al., 2006; Leterrier et al.,

2007). Recent research has examined and characterized some genes with increased expression in the pea genome under cold stress conditions (Lucau-Danila et al., 2012). Among these genes, *TOP2*, a topoisomerase involved in changing the topology of DNA in all processes associated with DNA metabolism such as replication, transcription, recombination, and chromosome segregation, and *PDH47*, a helicase enzyme with functions in DNA replication, repair, recombination, transcription, ribosome biogenesis, and translation initiation, have been identified (Hettiarachchi et al., 2005; Vashisht & Tuteja, 2006).

Among DNA topoisomerases, plant topoisomerases constitute the least studied enzyme group when compared to bacteria, yeast, and animal systems (Singh et al., 2004). Among plants *TOP2* gene sequences are known only in tobacco, pea and *Arabidopsis thaliana* (thale cress), which is a model plant in Brassicaceae family, for studying plant biology and genetics (Hacker et al., 2022). Recent studies have revealed that, in addition to its classical functions, the enzyme also plays a role in the relationship between DNA replication/cell cycle and abiotic stresses. It has been demonstrated that gene expression is regulated through light signal pathways mediated by phytochrome (Hettiarachchi et al., 2003). In another study, the expression of the *TOP2* gene in peas was increased under cold and salt stresses, as well as with applications of the phytohormones salicylic acid and ABA (Hettiarachchi et al., 2005).

Helicases, like topoisomerases, also play crucial roles in DNA metabolism. Most helicases belong to the DEAD-box protein family and are involved in fundamental cellular processes such as DNA replication, repair, recombination, transcription, ribosome biogenesis, and translation initiation (Vashisht & Tuteja, 2006). Compared to organisms like humans, flies, worms, and yeast, with sequenced genomes, the Arabidopsis plant boasts the highest number of DEAD-box RNA helicase genes, exceeding 50 (Boudet et al., 2001). In plants, the presence of DEAD-box helicase genes whose expression is regulated by cold and their involvement in stress signal transduction were first reported in *Arabidopsis* (Seki et al., 2001).

In peas, a DEAD-box DNA helicase gene named *PDH47* has been characterized, showing 93% similarity to the translation initiation factor eIF4A protein in tobacco. The exact function of helicases under stress conditions is not fully known, but it is believed that they regulate some stress-induced metabolic pathways by activating transcription factors, interact with topoisomerases, play regulatory roles in transcription and translation after being phosphorylated by protein kinases, and may have functions in the repair of damaged DNA/RNA molecules (Vashisht & Tuteja, 2006). The expression of the pea DEAD-box DNA helicase 47 gene has been found to significantly increase in stems and roots under salt and cold stress, but the gene did not respond to heat stress (Vashisht et al., 2005).

In recent years, silicon has been identified as one of the substances that have positive effects on the development of tolerance in plants under stress conditions (Tayade et al., 2022). Silicon ranks second among the most abundant elements in the Earth's crust (Raza et al., 2023). Plants obtain silicon from the soil through their roots in the form of monomeric molecule silicic acid [$Si(OH)_4$] when the pH is below 9 (Mandlik et al., 2020). Transportation of B from soil to plant roots takes place via passive diffusion as well as boron transporters NIP5;1 and BOR1, in addition to aquaporin proteins, which take role in transport of B across the plasma membranes (Takano et al. 2005; Fitzpatrick and Reid 2009). Silicon is not listed among the essential elements for plant development, and it is not present in nutrient media used in plant tissue cultures. This is because plants do not require this element for growth under optimum conditions (Epstein, 2009). However, recent studies have revealed the vital roles of silicon in the presence of biotic and abiotic stresses (Thakur et al., 2023; Shanmugaiah et al., 2023).

For example, the accumulation of silicon in roots reduces apoplastic bypass flow and provides binding sites for metals (Mostafa et al., 2021). Silicon reduces the uptake of toxic metals and salts into cells and their transmission from roots to stems, by competing selectively with transporter proteins (Mir et al., 2022). Silicon accumulation in leaves and stems helps strengthen cell walls and has a hardening effect, which protects the plants against strong winds and heavy rain (Collin et al., 2014; Zargar et al., 2019). A recent study used RNA sequencing analysis to reveal that silicon regulates the biosynthesis of alkaloids and flavonoids, helps maintenance of cellular redox homeostasis and osmotic adjustments, and promotes the deposition of complex carbohydrates in the cell wall (Biju et al., 2023). Additionally, silicon accumulation in leaves was shown to decrease transpiration in the cuticle, thereby enhancing resistance to low and high temperatures, drought stress, radiation, and UV stress in plants (Islam et al., 2020). The beneficial effects of silicon are found more pronounced in stem tissues (Ma & Yamaji, 2006).

It has been suggested that silicon stimulates the formation of defense components under stress and has affinities with some organic compounds (Bakhat et al., 2018). The resistance-enhancing effects of silicon under various stresses have also been demonstrated in various plants in recent studies. For example, in the powdery mildew-infected *Arabidopsis* plants, silicon induced the differential expression of various genes, while its presence did not affect gene expression in non-infected plants (Fauteux et al., 2006). In pea plants, silicon increased the production of chitinase and glucanase enzymes in tissues infected with leaf spot pathogenic fungi and accumulated significantly in the leaves (Dann & Muir, 2002).

Silicon has also shown its positive effects in cucumber, tomato, and canola plants under salt stress, reducing tissue reactive oxygen levels and increasing antioxidant system activity (Zhu et al., 2004; Al-Aghabary et al., 2005; Hasanuzzaman et al., 2018). Under cold stress, wheat, maize, and barley responded to silicon applications mainly by activating enzymatic and non-enzymatic antioxidant system components, increasing tissue water content, and significantly enhancing cold resistance (Liang et al., 2008; Moradtalab et al., 2018; Joudmand & Hajiboland, 2019). In a study where the Lsi1 gene responsible for transporting silicon to roots was overexpressed, rice plants showed cold resistance by maintaining osmotic balance, increasing calcium storage, and proline production (Xie et al., 2022).

Studies on silicon applications in pea plants mostly focused on the development and responses of peas under salinity and heavy metal stress. In these studies, silicon application has been found to enhance the main enzymatic and non-enzymatic antioxidant defense systems in peas, providing stress tolerance (Batool et al., 2022; El-Okkiah et al., 2022; Ismail et al., 2022; Oliveira et al., 2020; Rahman et al., 2017; Cruzado-Tafur et al., 2023; Salman et al., 2023).

To our knowledge, there is no information on the damage to the cell membrane in peas under cold stress and silicon application, as well as chlorophyll content, hydrogen peroxide (H_2O_2), and proline levels, and the enzymatic defense initiated by the superoxide dismutase (SOD) enzyme activity, which converts superoxide radicals to H_2O_2. In the scope of this study, various physiological and genetic responses of peas under cold and freezing stress were examined comparatively. Parameters such as stem/root length, dry/fresh weight, relative water content, proline, malondialdehyde, hydrogen peroxide contents, chlorophyll levels, ion leakage, and activities of the SOD enzyme, indicators of the antioxidant system, were investigated. Furthermore, gene expressions of a topoisomerase *TOP2*, which plays important roles in the replication, transcription, and repair of DNA, and *PDH47*, a DEAD-box DNA helicase gene, were examined in leaf tissues under cold and freezing stress in the presence of silicon. Thereby, the physiological and genetic responses of peas under cold and freezing stresses were analyzed comparatively, potential tolerance mechanisms were identified, and the role of silicon in regulating gene expressions and interacting with proline and SOD metabolic pathways were evaluated for potential agricultural applications.

Materials and Methods

In this study, the pea variety Emerald, developed and registered by Istanbul Seed Company was used.

Cold Stress Applications

Pea seeds were sterilized with a 2% sodium hypochlorite solution to prevent seed derived contamination in hydroponics solution. The lowest sodium hypochlorite concentration that ensured sterility without reducing germination rate was determined as 2%, as a result of our preliminary experiments on pea. The seeds were germinated in 200 ml polypropylene containers containing sterile perlite, with three seeds each, and irrigated with ½ Hoagland solution (Hoagland & Arnon, 1950) every three days. The seedling development continued for 15 days in a plant growth chamber (Nuve GC 400, Turkey) with a 16-hour light/8-hour dark cycle, at 23°C, and 50% humidity, to imitate long days of late spring and early summer. Cold stress was applied by lowering the temperature to 4°C after 15 days, and samples for analysis were collected on the 1st and 4th days of cold stress (24 hours and 96 hours after the initiation of cold stress). Freezing stress was induced by lowering the temperature to -0.5°C, and samples were collected during the same periods as cold stress. Plants treated with silicon dioxide (at concentrations of 1 and 2 mM) were germinated by irrigating with ½ Hoagland solution containing silicon dioxide of the specified concentrations as soon as they were placed on perlite. The concentration of silicon dioxide was determined based on previous hydroponic studies in plant-silicon literature (Parveen & Ashraf 2010; Zhang et al., 2011). Cold and freezing stresses, as well as sample collection periods, were applied as described above. Control plants kept under normal conditions spent an additional 1 and 4 days in the same environment after the 15-day germination period, ensuring that they experienced the same duration as the stressed plants in the culture environments.

All treatments were performed in triplicate, with each replicate consisting of four polypropylene containers containing three seeds each. This resulted in 12 pea seedlings prepared per treatment, and with replicates, there were a total of 36 seedlings for each treatment.

Determination of Stem/Root Length and Relative Water Content

After completing 15 days of development, plants subjected to stress with or without the presence of silicon dioxide, along with control plants, were uprooted from perlite, washed under tap water, and root and stem lengths were determined. Root and stem tissues were separated, weighed, and after drying at 60°C for 48 hours, weighed again to determine dry weights. Relative water content (RWC) was calculated according to the formula RWC(%) = (Fresh weight - Dry weight) / (Turgid weight - Dry weight) X 100, from Smart and Bingham (1974). Turgid weight was calculated by soaking tissues in distilled water at room temperature for 24 hours.

Determination of Proline Content

The determination of proline content started with the homogenization of 0.3 g leaf samples in liquid nitrogen, followed by dissolution in 1 ml of 3% sulfosalicylic acid (Bates et al., 1973). Then, 0.1 ml of the sample, after centrifugation, was mixed with 0.2 ml acid ninhydrin, 0.2 ml of 96% acetic acid, and 0.1 ml of 3% sulfosalicylic acid. The mixtures were held at 96°C for 1 hour, and after centrifugation and mixing with 1 ml of toluene, the absorbance of the upper phase was read at 520 nm.

Determination of Hydrogen Peroxide Content

The amounts to be determined according to Bergmeyer (2012) started with the liquid nitrogen homogenization of 0.5 g leaf tissue and dissolution in 1.5 ml of 100 mM potassium phosphate buffer (pH 6.8). In the samples where 0.25 ml of supernatant was collected after centrifugation, the enzymatic reaction was initiated by mixing with 1.25 ml peroxidase solution (83 mM potassium phosphate buffer, pH 7.0, 0.005% (w/v) o-dianisidine, 40 µg peroxidase/ml) at 30°C. After 10 minutes, the reaction was stopped by adding 0.25 ml of 1 N perchloric acid, and the absorbance of the supernatant was measured at 436 nm after centrifugation.

Determination of Malondialdehyde (MDA) Content

The determination of MDA content for assessing cell membrane damage followed the Ohkawa et al. (1979) method. The homogenization of 0.2 g leaf tissue in liquid nitrogen was followed by adding 1 ml of 5% trichloroacetic acid (TCA). After centrifugation, the same volume of 20% TCA containing 0.5% thiobarbituric acid (TBA) was added,

and the mixture was kept at 96°C for 25 minutes. The samples were cooled on ice, and the absorbance was read at 532 nm. Non-specific absorbance at 600 nm was determined and subtracted from the initial absorbance value.

Ion Leakage

Ion leakage, determined according to the method of Nanjo et al. (1999), involved shaking six leaves in 15 ml tubes containing 5 ml of 0.4 M mannitol. Electrical conductivity was recorded as C1 using a Mettler Toledo MPC 227 conductivity meter. After boiling for 15 minutes and cooling the samples to room temperature, C2 readings were taken, and the leakage-related conductivity was calculated using the formula [(C1/C2) X 100].

Determination of Chlorophyll Content

Chlorophyll amounts were determined using the method of Lichtenthaler and Wellburn (1983). This involved the liquid nitrogen homogenization of 3 g leaf tissue, centrifugation, and determination of the supernatant at different absorbance values. Chlorophyll a (mg/L) and chlorophyll b (mg/L) were calculated using the following formulas:

- Chlorophyll a (mg/L) = 15.65 Abs666 – 7.340 Abs653
- Chlorophyll b (mg/L) = 27.05 Abs653 – 11.21 Abs666

Determination of Superoxide Dismutase (SOD) Enzyme Activities

Enzyme activities were determined according to the method of Beauchamp and Fridovich (1971). The homogenization of 0.2 g leaf samples in a glass-glass homogenizer mixed with homogenization buffer on ice was followed by centrifugation, and the supernatant was stored at -80°C until use. The protein content of the extracts was determined by the Bradford (1976) method. A native polyacrylamide gel consisting of a separation part and stacking part with a 30% (29:1) acrylamide-bis solution was prepared for use in a Bio-Rad midi gel apparatus. After loading the samples, electrophoresis was conducted at 8 V/cm, monitoring the tracking dye. Subsequently, the gel apparatus was disassembled, and for the determination of different isozymes of the SOD enzyme, first KCN and hydrogen peroxide were applied, and then negative activity staining with NBT was performed to visualize the enzyme isozyme bands.

Determination of Gene Expressions for TOP2 and PDH47 Genes

The mRNA sequences of the *TOP2* and *PDH47* genes with GenBank accession numbers Y14559.1 and AY167670.1 were obtained from the National Center For Biotechnology Information (NCBI) database. The expression at the transcription level of these genes was investigated using the semi-quantitative reverse-transcription PCR (RT-PCR) technique. The pea actin gene with the GenBank accession number X68649.1 was used as an internal control.

RNA Isolation and Reverse Transcription PCR (RT-PCR)

RNA isolation from pea leaves was performed using Qiagen RNeasy plant mini kits based on guanidine-isothiocyanate lysis and silica-membrane purification methods. The quantity of the obtained total RNAs was determined spectrophotometrically, and their quality was assessed by separating and visualizing them with 2% agarose gel electrophoresis. cDNA libraries were created using the Thermo First Strand cDNA Synthesis Kit (Thermo, USA) from the obtained RNA molecules. From this library, the *TOP2* and *PDH47* genes with NCBI accession numbers Y14559.1 and AY167670.1 were amplified by PCR using primers designed with the PrimerPremier 5.0 program from CA, USA, providing the most suitable conditions for amplification. The obtained bands were separated on a 0.8% agarose gel and visualized using the Biolab UV Tech gel imaging system. The bands were analyzed numerically using the ImageJ software developed by the National Institute of Health (NIH, USA) to determine differences in gene expression levels.

Statistical Analyses

The data obtained in the study were evaluated using the SPSS 16.0 program. Differences between applications were determined by comparing means with the One Way Anova and Tukey Test.

Results and Discussion

Pea plants exposed to cold and freezing stresses exhibited different morphological features depending on the presence of silicon in the environment, and the water-holding capacity of leaves also changed (Table 1). The stem length of the plants decreased with the intensity of stress on the 4th day, while the presence of silicon did not help length recovery. Root lengths did not respond to the presence of stress or silicon, except showing significant increases upon silicon applications under normal growth conditions.

The only treatment altering the relative water content compared to control plants is the 4-day freezing stress application. Under this treatment, the leaf water holding capacity of control plants decreased; however, both concentrations of silicon significantly increased water holding capacity. The application that most notably affected water holding capacity was 2mM silicon, showing a 15% increase compared to the control. The dry weights of stems and roots varied between 0.27-0.32g and 0.19-0.25g, respectively, under different treatments, but none of the applications had a significant effect on tissue dry weights. Therefore, these results have not been included in the Table 1.

Low ion leakage levels, an indicator of cell integrity, demonstrated that tissues under silicon applications suffered less damage compared to the control in both root and stem tissues and at all applied temperature values (Table 2). While similar decreases were observed under all treatments, statistically significant decreases in ion leakage were determined as follows: in 1-day stem tissues under normal conditions with 1mM silicon application, in 4-day stem tissues under normal conditions with 2mM silicon application, in stem tissues after 1-day cold stress compared to the control under all applications, in stem tissues after 4-day freezing stress compared to the control under all applications, and in stem tissues under 1mM silicon application during 1-day freezing stress. A similar situation was observed in root tissues, and statistically significant decreases were determined in 1-day cold stress with 1mM silicon application, in 1-day freezing stress with 2mM silicon application, and 4-day freezing stress compared to the control under all silicon applications.

Table 1. Plant morphological responses and leaf relative water content (RWC).

Treatments	Relative Water Content (%)	Shoot Lenght (cm)	Root Lenght (cm)
1st day of stress			
C*	99 ± 0.7	5.74 ± 0.22	$8.36 \pm 0.44_a$
S1	99 ± 1.2	5.97 ± 0.40	$10.04 \pm 0.42_b$
S2	100 ± 0.7	6.51 ± 0.32	$11.14 \pm 0.69_b$
C st4**	99 ± 1.1	5.21 ± 0.24	8.69 ± 0.58
S1 st4	100 ± 0.3	6.50 ± 0.21	9.68 ± 0.37
S2 st4	96 ± 0.4	6.32 ± 0.51	9.71 ± 0.40
C st-	99 ± 0.7	4.40 ± 0.25	8.92 ± 0.48
S1 st-	100 ± 0.6	4.51 ± 0.23	8.91 ± 0.20
S2 st-	99 ± 1.3	4.91 ± 0.21	8.03 ± 0.37
4th day of stress			
C	95 ± 1.2	7.35 ± 0.46	$9.72 \pm 0.2_a$
S1	100 ± 0.7	7.72 ± 0.29	$10.77 \pm 0.27_b$
S2	100 ± 0.3	7.62 ± 0. 32	$11.00 \pm 0.31_b$
C st4	100 ± 0.5	5.32 ± 0.27	7.61 ± 0.34
S1 st4	97 ± 1.6	5.23 ± 0.16	8.41 ± 0.30
S2 st4	100 ± 0.9	5.20 ± 0.35	8.47 ± 0.46
C st-	$66 \pm 1.8_a$	4.88 ± 0.44	9.28 ± 0.51
S1 st-	$73 \pm 1.9_b$	5.46 ± 0.53	10.02 ± 0.64
S2 st-	$81 \pm 1.2_b$	4.61 ± 0.45	8.98 ± 0.39

*C, S1 and S2 represent plants irrigated with Hoagland solution containing 0mM, 1mM and 2mM silicon in nutrient media, respectively. ** The abbreviations next to the treatments; st4 represents cold stress applied at 4°C, and st- represents freezing stress applied at -0.5°C; The letters at the bottom right of the results indicate statistically significant differences ($p \leq 0.05$) compared to the respective control. No significant difference is present where no letter was indicated within the treatment groups (C. S1. S2).

Table 2. Ion leakage. malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) levels in silicone-applied pea tissues under cold and freezing stress and normal conditions.

Treatments	Ion Leakage (%)		Malondialdehyde Content (nmol/g)		H_2O_2 Content (nmol/g)	
1st day of stress						
	Shoot	Root	Shoot	Root	Shoot	Root
C*	$21.16 \pm 1.97_a$	40.07 ± 2.96	0.019 ± 0.001	$0.012 \pm 0{,}001_a$	$0.26 \pm 0.03_a$	$0.37 \pm 0.04_a$
S1	$15.03 \pm 0.56_b$	35.51 ± 2.67	0.018 ± 0.000	$0.009 \pm 0.000_a$	$0.10 \pm 0.02_b$	$0.31 \pm 0.04_b$
S2	$17.57 \pm 1.92_a$	32.30 ± 2.89	0.018 ± 0.002	$0.008 \pm 0.001_b$	$0.12 \pm 0.01_b$	$0.23 \pm 0.02_b$
C st4**	$19.58 \pm 1.26_a$	$31.36 \pm 3.30_a$	0.020 ± 0.002	0.014 ± 0.001	$0.46 \pm 0.07_a$	$0.49 \pm 0.04_a$
S1 st4	$15.6 \pm 0.97_b$	$23.14 \pm 1.32_b$	0.021 ± 0.002	0.013 ± 0.000	$0.21 \pm 0.03_b$	$0.29 \pm 0.01_b$
S2 st4	$14.26 \pm 1.08_b$	$27.15 \pm 2.86_a$	0.021 ± 0.001	0.013 ± 0.001	$0.24 \pm 0.03_b$	$0.22 \pm 0.00_b$
C st-	$17.34 \pm 1.94_a$	$16.88 \pm 1.40_a$	$0.018 \pm 0.001_a$	$0.018 \pm 0.002_a$	0.37 ± 0.02	$0.50 \pm 0.06_a$
S1 st-	$13.06 \pm 0.64_b$	$13.84 \pm 0.56_a$	$0.018 \pm 0.001_a$	$0.013 \pm 0.000_b$	0.30 ± 0.04	$0.30 \pm 0.04_b$
S2 st-	$13.81 \pm 1.73_a$	$10.90 \pm 0.83_b$	$0.012 \pm 0.002_b$	$0.012 \pm 0.001_b$	0.29 ± 0.02	$0.20 \pm 0.03_b$
4th day of stress						
	Shoot	Root	Shoot	Root	Shoot	Root
C	$25.66 \pm 3.21_a$	34.07 ± 3.79	0.018 ± 0.002	0.010 ± 0.000	$0.32 \pm 0.02_a$	$0.49 \pm 0.04_a$
S1	$22.82 \pm 2.75_a$	31.42 ± 2.76	0.015 ± 0.001	0.008 ± 0.000	$0.25 \pm 0.02_b$	$0.38 \pm 0.03_b$
S2	$18.73 \pm 2.06_b$	27.82 ± 2.16	0.015 ± 0.001	0.008 ± 0.000	$0.24 \pm 0.02_b$	$0.24 \pm 0.01_b$
C st4	23.45 ± 2.22	37.33 ± 2.73	0.021 ± 0.001	$0.018 \pm 0.001_a$	$0.45 \pm 0.03_a$	$0.51 \pm 0.03_a$
S1 st4	22.46 ± 1.78	37.3 ± 3.18	0.021 ± 0.002	$0.013 \pm 0.001_b$	$0.32 \pm 0.01_b$	$0.33 \pm 0.01_b$
S2 st4	23.26 ± 2.74	36.22 ± 2.41	0.019 ± 0.001	$0.013 \pm 0.000_b$	$0.18 \pm 0.02_b$	$0.39 \pm 0.03_b$
C st-	$20.12 \pm 1.79_a$	$24.20 \pm 1.46_a$	0.014 ± 0.000	$0.012 \pm 0.001_a$	$0.25 \pm 0.03_a$	$0.68 \pm 0.05_a$
S1 st-	$13.59 \pm 1.04_b$	$20.03 \pm 1.83_b$	0.012 ± 0.002	$0.012 \pm 0.001_a$	$0.20 \pm 0.00_b$	$0.47 \pm 0.02_b$
S2 st-	$11.75 \pm 1.00_b$	$17.25 \pm 0.69_b$	0.012 ± 0.001	$0.007 \pm 0.000_b$	$0.20 \pm 0.01_b$	$0.31 \pm 0.02_b$

*C, S1 and S2 represent plants irrigated with Hoagland solution containing 0mM, 1mM and 2mM silicon in nutrient media, respectively. ** The abbreviations next to the treatments; st4 represents cold stress applied at 4°C, and st- represents freezing stress applied at -0.5°C; The letters at the bottom right of the results indicate statistically significant differences ($p \leq 0.05$) compared to the respective control. No significant difference is present where no letter was indicated within the treatment groups (C. S1. S2).

MDA levels, an indicator of cell membrane damage, showed a decrease in stem tissues on the 1st day of freezing stress with 2mM silicon application compared to the control. MDA levels, which remained the same under all treatments under normal conditions, exhibited significant decreases, especially in root tissues, with silicon application under cold stress. When root tissues were examined, it was observed that even under normal conditions, the 2mM silicon application reduced tissue MDA levels, and under 4-day cold stress, silicon

applications effectively reduced MDA levels. Under freezing stress, a 1-day application with two different silicon concentrations and a 4-day application with 2mM silicon reduced MDA levels in root tissues.

Even without any stress application, silicon concentrations on the 1st day in pea tissues kept hydrogen peroxide levels lower than in other tissues. During the 4-day culture stage, tissue hydrogen peroxide levels remained low at all silicon concentrations. Under cold stress, all silicon concentrations effectively reduced hydrogen peroxide levels in both root and stem tissues. Under freezing stress, silicon applications had a level-reducing effect on stem tissues for the 4-day duration, while in root tissues, silicon concentrations reduced hydrogen peroxide levels compared to other applications under all stress durations.

On the first day of cold stress, chlorophyll levels increased compared to applications without cold stress, while under freezing stress, they decreased both under normal conditions and compared to cold stress (Table 4). Silicon applications did not alter chlorophyll levels in tissues under any stress condition. Osmoprotectant proline levels, which play a role in stress resistance, remained lower in stem tissues even under 1-day normal conditions with silicon applications compared to the control, with no significant differences observed among applications in root tissues. Both in root and stem tissues, silicon concentrations under 1 and 4 days of cold stress caused a significant decrease in proline levels. Under freezing stress, silicon applications reduced proline levels in both stem and root tissues.

Table 3. Chlorophyll a, chlorophyll b and proline levels in silicone-applied pea tissues under cold and freezing stress and normal conditions.

Treatments	Chlorophyll Content (µg/g)		Proline Content (nmol/g)	
	1st day of stress			
	Ca	Cb	Shoot	Root
C*	15.17 ± 1.87	9.05 ± 1.02	$188.66 \pm 13.21_a$	73.20 ± 6.71
S1	14.70 ± 1.65	9.03 ± 0.08	$138.32 \pm 13.75_b$	74.74 ± 6.64
S2	15.86 ± 1.24	9.31 ± 0.07	$147.42 \pm 12.02_b$	81.70 ± 7.20
C st4**	18.68 ± 1.89	11.50 ± 1.23	$463.40 \pm 16.21_a$	$102.31 \pm 9.89_a$
S1 st4	19.59 ± 2.22	11.71 ± 1.12	$189.00 \pm 13.89_b$	$75.26 \pm 7.23_b$
S2 st4	18.95 ± 1.98	11.60 ± 1.06	$173.71 \pm 14.15_b$	$62.11 \pm 5.36_b$
K st-	8.22 ± 1.12	7.83 ± 0.07	$208.59 \pm 14.33_a$	$149.14 \pm 13.15_a$
S1 st-	8.23 ± 0.08	7.47 ± 0.06	$194.59 \pm 12.86_a$	$82.47 \pm 13.11_b$
S2 st-	7.99 ± 0.09	7.21 ± 0.08	$132.47 \pm 13.42_b$	$85.05 \pm 12.78_b$
	4th day of stress			
	Ca	Cb	Shoot	Root
C	19.35 ± 1.43	11.80 ± 1.78	145.53 ± 12.99	73.54 ± 9.29
S1	19.15 ± 2.13	11.45 ± 0.87	142.84 ± 13.21	70.10 ± 6.14
S2	20.27 ± 1.29	11.02 ± 1.03	140.52 ± 12.93	69.93 ± 5.96
C st4	19.32 ± 2.06	11.31 ± 1.33	$418.30 \pm 17.32_a$	$106.96 \pm 11.69_a$
S1 st4	19.62 ± 1.92	11.58 ± 1.85	$248.97 \pm 14.66_b$	$76.80 \pm 5.12_b$
S2 st4	20.69 ± 1.69	12.56 ± 1.98	$202.58 \pm 13.51_b$	$85.05 \pm 6.60_b$
C st-	8.34 ± 0.34	8.69 ± 0.60	$274.23 \pm 14.47_a$	99.48 ± 8.34
S1 st-	9.01 ± 1.08	7.64 ± 0.71	$163.40 \pm 13.95_b$	87.29 ± 8.03
S2 st-	8.68 ± 0.44	7.70 ± 0.63	$192.78 \pm 12.38_b$	86.08 ± 7.72

*C, S1 and S2 represent plants irrigated with Hoagland solution containing 0mM, 1mM and 2mM silicon in nutrient media, respectively. ** The abbreviations next to the treatments; st4 represents cold stress applied at 4°C, and st- represents freezing stress applied at -0.5°C; The letters at the bottom right of the results indicate statistically significant differences ($p \leq 0.05$) compared to the respective control. No significant difference is present where no letter was indicated within the treatment groups (C. S1. S2).

Table 4. Silicon accumulation in stem and root tissues under normal conditions with cold and freezing stress.

Treatments	Amount of Silicon (mg/kg dry weight)			
	1st day of stress		4th day of stress	
	Shoot	Root	Shoot	Root
S1	$257.5 \pm 7.52_a$	$264.8 \pm 2.85_a$	$327.2 \pm 1.86_{a,\,b}$	$1430 \pm 35.8_a$
S2	$359.9 \pm 4.17_b$	$550.2 \pm 36.42_c$	$470.9 \pm 14.61_c$	$1455 \pm 24_a$
S1 st4*	$282.7 \pm 7.15_a$	$633 \pm 21.22_d$	$346.1 \pm 10.49_{a,\,b}$	$1782 \pm 21.54_b$
S2 st4	$339.5 \pm 32.75_b$	$809 \pm 17.19_e$	$523.8 \pm 12.50_d$	$1809 \pm 39.41_b$
S1 st-	$279.2 \pm 12.77_a$	$408.1 \pm 6.98_b$	$320.8 \pm 0.58_a$	$916.1 \pm 40.86_c$
S2 st-	$338.2 \pm 15.38_b$	$573.8 \pm 12.25_c$	$347.4 \pm 7.05_b$	$1258 \pm 40.6_d$

*C, S1 and S2 represent plants irrigated with Hoagland solution containing 0mM, 1mM and 2mM silicon in nutrient media, respectively. ** The abbreviations next to the treatments; st4 represents cold stress applied at 4°C, and st- represents freezing stress applied at -0.5°C; Different letters at the bottom right of the results indicate statistically significant differences ($p \leq 0.05$) compared to the respective control in the same column.

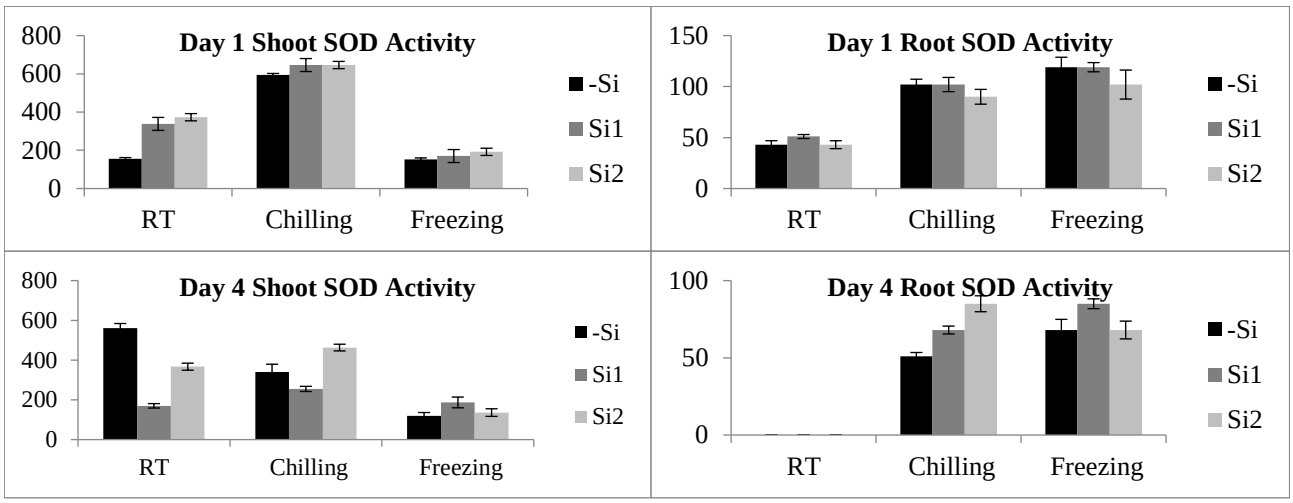

Figure 1. Total SOD enzyme activity of shoot and root tissues under different silicon and stress applications

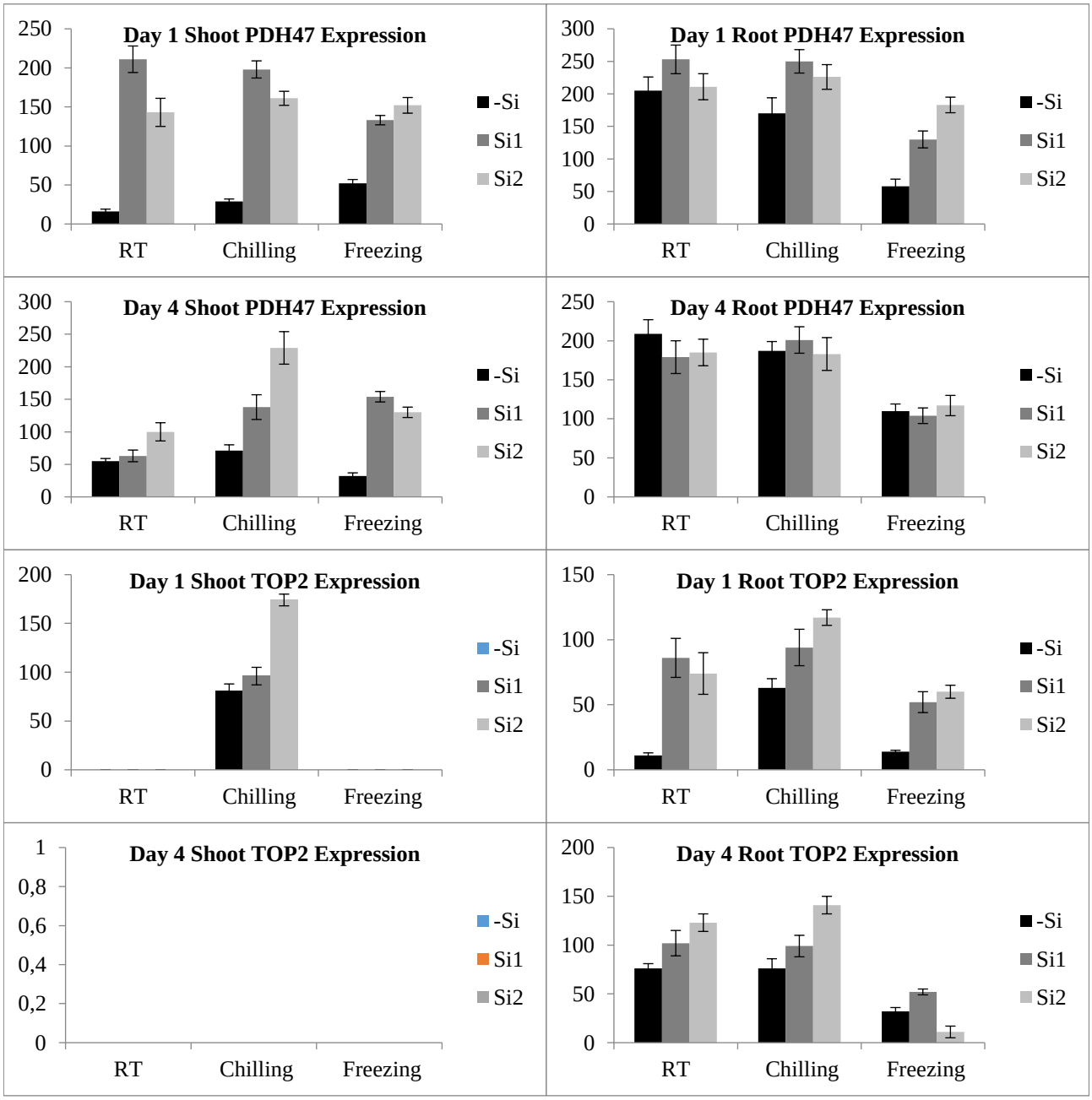

Figure 2. The colors of Si treatments should be black, dark gray and light gray as other graphs

SOD isoenzyme activity was examined using the non-denaturing PAGE method. While three different isoenzyme activities were observed in leaves, one or two different isoenzyme activities were observed in roots under different treatments. The isoenzyme types were determined to be Cu/ZnSOD and MnSOD using KCN and H_2O_2. Isoenzyme activities were determined by examining band thicknesses formed under different treatments, and it was observed that different isoenzyme activities could appear in different tissues under different temperature applications. For example, MnSOD and CuZnSOD1 activities were observed in roots only under freezing stress, CuZnSOD2 isoenzyme activity was observed on the 1st day under all temperature applications, and on the 4th day, it was only evident in root tissues under cold stress. In stem tissues, the activities of all three SOD isoenzymes were observed under every condition. In root tissues, the application of 1mM silicon on the 4th day of cold and freezing stress increased the activity of the SOD enzyme (CuZnSOD2).

In stem tissues, on the 1st day under normal conditions, SOD activity belonging to three different isoenzymes increased in all applications compared to the control, while under normal conditions on the 4th day, the same applications decreased all isoenzyme activities compared to the control (Figure 1). Under cold stress on the 1st day, two different silicon concentrations increased SOD enzyme activity in stem tissues. On the fourth day of cold stress, under 2mM silicon applications, the activity in stem tissues increased. Under freezing stress, all applications increased total SOD activity in the form of CuZnSOD2 in the stem on both the 1st and 4th stress days, and 1mM silicon increased all isoenzyme activities on the 4th stress day.

Among the genes examined for expression levels under stress conditions and various substance applications in the study, *TOP2*, exhibited expression only in stem tissues under 1-day cold stress, and its expression increased with silicon applications (Figure 2). The *TOP2* gene activity was observed in root tissues for every application, and its expression intensity increased in the presence of silicon in all applications, including normal conditions on the 1st day, and on the 4th stress day, both under normal conditions and cold stress.

In the absence of any treatments, the *PDH47* gene expression in pea roots was more intense compared to stems (Figure 2). *PDH47* gene expression increased in all treatments, with an increase in all concentrations of silicon present in the environment. Although the expression of *PDH47* in root tissues was not as intense as in stem tissues, it increased in response to all silicon applications except for the 4th freezing stress day. The expression of the *PDH47* gene in stem tissues increased under cold stress, with a decrease observed only on the 4th day under freezing stress compared to the control. *PDH47* gene expression decreased in root tissues under both cold and freezing stress. Actin expression decreased under both cold and freezing stresses in both root and stem tissues, except for the stem tissues on the 4th day under cold stress.

Elemental silicon analysis in pea tissues was conducted using the Perkin Elmer OPTIMA 5300 DY ICP OES device, following the EPA 6010 method. As expected, silicon uptake into tissues from environments containing 2mM silicon was greater than those containing 1mM silicon (Table 4). The obtained data revealed that silicon accumulation in root tissues showed increases up to fivefold compared to the stem. These data suggest that silicon uptake into cells via silicon carriers is an efficient mechanism, but it is not transmitted to the stem at the same rate as vascular tissues. The most notable finding from elemental analysis was the higher concentration of silicon in root tissues under cold stress compared to the control. This condition persisted on the 1st day under freezing stress, but on the 4th day of freezing stress, silicon uptake into cells did not continue at the same pace.

In summary, under cold and freezing stress, the dry weights of pea roots and stems remained unchanged independent of cold stress and silicon applications. Different temperature applications did not have a significant effect on root lengths on the 1st and 4th day of stress. Shoot tissue was also not affected on the 1st day of stresses, however on the 4th day, stems under cold and freezing stress were shorter than those developing under normal conditions. Sogarwal et al. (2023) demonstrated that exogenous Si application in wheat increased cuticle and epidermal layer thickness due to accumulated silicon, preventing the harmful effects of drought, heat, and cold stress. In the context of this study, root lengths were observed to be extended in the presence of silicon under normal growth conditions compared to the control; however, no significant effect of silicon presence were observed in tissue weights and lengths under the investigated stress conditions, likely due to stress intensity and difference in plant response used in this particular study.

When all indicators of oxidative stress levels were compared, it was determined that silicon played an important role in preserving cell membrane integrity, thereby reducing ion leakage and lipid peroxidation while increasing water retention capacity. Additionally, silicon significantly reduced hydrogen peroxide levels in tissues, lowering the levels of accumulated reactive oxygen species in cells. In stressed tissues, especially under cold stress, proline levels increased significantly; however, silicon did not further increase proline levels under stress conditions. Instead the levels decreased under stress upon silicon application. Studies conducted on turfgrass and corn plants have shown that silicon application increases tissue proline levels under cold stress (He et al., 2010; Moradtalab et al., 2018). In our study, silicon likely contributed to a decrease in stress levels by reducing internal reactive oxygen species and, consequently, proline levels. This observation suggests that different physiological responses may be obtained in different plants depending on stress duration and dosage.

The findings of this study indicated that SOD enzyme activity, especially on the 1st day of cold stress, showed significant increases compared to plants under normal conditions, but as stress conditions prolonged or intensified in the form of freezing stress, activities in stems decreased. Pea seedlings adapted to short periods of low-intensity cold stress in stem tissues through enzymatic antioxidant defense proteins, but they could not withstand prolonged severe cold stress likely due to possible structural and functional impairments in defense system components. However, SOD enzyme activities continued intensively in root tissues under both cold and freezing stress, with

significant increases observed in the presence of silicon at different enzyme isoform levels. Similar protective effects of silicon on SOD enzyme and other enzymatic antioxidants have also been observed in studies on paspalum turfgrass, rice, corn, and barley (He et al., 2010; Azeem et al., 2016; Moradtalab et al., 2018; Joudmand & Hajiboland, 2019).

Under cold and freezing stresses, our examinations with silicon applications revealed that *TOP2*, *PDH47*, and actin gene expressions were activated by stress, indicating a response to the presence of silicon in the environment. The obtained data suggest that *TOP2*, which is one of the topoisomerase enzyme genes involved in DNA metabolism processes such as replication, transcription, recombination, and chromosome segregation, starts expressing on the first day of cold stress, despite not being expressed under normal conditions. This implies that *TOP2* begins to alter the topology of DNA, potentially affecting various stress-related genes and transcription factors involved in replication, transcription, recombination, and chromosome segregation.

The presence of silicon in the environment significantly increased the expression of *TOP2*. Hettiarachchi et al. (2005) also demonstrated an increase in *TOP2* gene expression under cold and salt stresses, as well as with applications of salicylic acid and ABA from phytohormones. The *PDH47* gene showed significant increases in expression in stem and root tissues under salinity and cold stress in peas (Vashisht et al., 2005). When the *PDH47* gene was transferred to indica rice, the plant exhibited resistance features under drought stress, as evidenced by increased relative water content with proline and reduced internal hydrogen peroxide levels (Singha et al., 2020; Singha et al., 2017). Although the reason for the increase in *TOP2* gene expression under stress, when DNA replication and cellular activities decrease, has not been fully explained, it has been suggested that it may be related to chromatin modeling and the necessity for DNA to adopt an appropriate topology for the expression of stress-regulated genes.

The data obtained in this study also suggest that the expressions of genes involved in ribosome biogenesis, transcription, translation, and repair of damaged DNA/RNA molecules, which are considered to have functions in stress tolerance, can be enhanced by silicon. These increases are believed to have positive effects on the antioxidative defense system. Silicon applications in pea stem tissues also increased the expression of the actin gene, which is investigated as an internal control under different temperatures. Changes in the expression of the actin protein, which structures the intracellular skeleton system, indicate that silicon may have protective functions related to the skeletal system.

The results of elemental silicon analysis have revealed an increase in the amount of silicon taken up by tissues at high silicon concentrations. It has also been found that roots contain a higher amount of silicon compared to stems, and the transport of silicon into the tissues increases under cold stress conditions. These data may explain the observed increase in root lengths and unaffected stem lengths under normal conditions with silicon application, as well as the decrease in malondialdehyde levels, which was only observed in roots under cold stress conditions with silicon applications.

Conclusions

This study has demonstrated that the applications of silicon to pea plants, when externally added to the environment in which they are cultivated, have positive effects on the cold resistance of plants. The obtained data have shown that silicon increases the expressions of *TOP2* and *PDH47* genes, which can alter the structure of the DNA molecule and facilitate the transcription of genes encoding defense enzymes, under both cold and freezing stresses. SOD enzyme activity, one of the most important enzymes used in combating reactive oxygen species, by converting superoxide radicals to hydrogen peroxide, increased under stress conditions and in the presence of silicon. The observed decrease in tissue hydrogen peroxide levels also indicates the active involvement of other components of the enzymatic defense system. Therefore, within the scope of this study, it can be concluded that silicon application under cold and freezing stresses has induced various changes in pea metabolism, including, an increase in the expressions of *TOP2* and *PDH47* genes and an increase in SOD enzyme activity, which in turn decreased tissue hydrogen peroxide levels and increased cell membrane integrity. Overall the changes have been found to have positive effects on the plants cold and freezing stress responses and silicon was determined as a potential soil amendment to be used in agricultural production.

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Nutritional Values of Partially Replacing the Commercial Soybean Meal by Raw, Full-Fat Soybean in Diets of Layers

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ABSTRACT

The aim of this study was to investigating the effects of partially replacing the commercial soybean meal (SBM) by locally produced raw, full-fat soybean (RFFSB) in diets of layers. After cleaning, the tested ingredient (RFFSB) was hammered to pass through a 0.2-mm sieve. Then, four experimental diets were formulated by replacing the SBM by RFFSB at 0, 15, 30 or 45% (equivalents to 0, 30, 60 or 90 g/kg of diet, respectively). Before the commencement of this feeding trial, birds were uniformly managed and fed as per their requirements (i.e., starter, grower and pullet diets). This feeding trial was started when birds' age was 24 weeks. Every treatment was replicated 4 times and 17 laying birds per replicate. The results revealed that replacing the commercial SBM by raw soybean (up to 45%) in the layer diets had no negative effects on the final live BWT and also on the vital organ developments, such as pancreas, duodenum, intestines and gizzard. Hen-day egg production, hen-housed egg production and egg quality measuring parameters were not significant affected by that of partially replacing the commercially SBM by the raw soybean. It is concluded that without compromising the productivity and health, a hammered RFFSB can replace (up to 45%) the commercial SBM in diets of the laying hens.

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Introduction

Progress of chicken production as well as egg consumption, in developing countries, including Ethiopia has not yet been as expected. Lack of quality and quantity of chicken feeds, mainly lack of the protein-source ingredient is one of the constraints for such low chicken production and productivity. As reviewed by Erdaw et al. (2016), commercial soybean meal (SBM), which is one of the best protein-source ingredients, is available as a byproduct (after extracting the oil) or as a full-fat product (without extracting the oil). Full-fat soybean can also be prepared from the raw soybean or after heating. The raw full-fat soybean (RFFSB), however, contains a variety of anti-nutritional factors (ANFs) which can impair the proper utilization of nutrients in soybeans by animals, particularly by the mono-gastric animals, in general and by the chickens, in particular (Mammo, 2019).

Protease inhibitors, lectins and phytate are those properly characterized ANFs in the raw soybean seed, so far (Pettersson and Pontoppidan, 2013). Protease (trypsin) inhibitors can interfere with the biological activity of an endogenous protease and thereby reduce the digestion of proteins (Nahashon and KilonzoNthenge, 2013). A nutritive value of RFFSB is negatively affected by the presence of ANFs (Liu et al., 1998; Erdaw and Beyene, 2018; Mammo, 2023), especially by trypsin inhibitors and lectins. As reported by Erdaw (2016) seed of raw soybean contains around 13498 rypsin inhibitor (TIU/g) as compared to 5743 (TIU/g) for the commercial SBM.

Consumption of the raw beans, including soybean beans increased the size of the pancreas and the duodenum and reduced feed intake and growth of the chicks. Scholars (Mogridge et al., 1996; ASA, 2004; Erdaw et al., 2019) reported that diets, containing raw beans reduced the feed consumption and live weight and also decreased the feed conversion indices.

The raw soybean inclusion, with share of 8% in the mixtures significantly reduced the number of eggs laid (Petričević et al., 2014). Differences, in body weights, food consumption, occurrence of defective eggs and the relative weight of the pancreas were not significantly influenced by feeding a graded levels of the two varieties of raw soybeans or by their interaction effects (Petričević et al., 2014).

Only the hens fed 20% of raw soybeans (i.e., this was the highest included level in the trial) in the diet laid

significantly fewer eggs than hens fed no raw soybeans (Latshaw & Clayton, 1976). As the percentage of raw soybeans increased, in the diet, there was a trend towards to the lower egg weight and body weight gains. Pancreas weights increased at an increasing rate of raw soybeans in diets (Latshaw & Clayton, 1976). However, ASA (2004) reported that whenever the age of the chicken advanced their tolerance to the ANFs is increasing.

Locally produced raw, full-fat soybean (grains) is cheaper than the commercial soybean meal (SBM) (i.e., from an informal discussion as well as observations, and from the farmers' workshops). The expensivenessof SBM is further exaggerated in some pocket-areas and also in the places where there are no sufficient oil-extracting plants. Sometimes, a double transportation cost is incurring against the producers. This means, firstly when transporting the raw soybean grain towards to the oil-extracting plant, and also when returning it (i.e., SBM), as the byproducts, back to the chicken producers, as feed. The oil-extracting plants are mostly installed near to the big-cites, which are actually far from the major grain-producing areas. Because of this all facts, this study was, therefore planned to investigate the effects of partially replacing the commercial soybean meal by that of locally produced raw, full fat soybean, and thereby, at least to reduce the feed-cost of laying (matured) hens.

Objective

Evaluate the effects of replacing commercial SBM by raw soybean in diets of layers

Materials and Methods

Tested Ingredient Preparation

Firstly, this feeding trial was approved by Animal Research Ethical Review Committee (Certificate Ref: No: VM/ERC/45/ 03/15/2023), and this trial was also executed in the poultry-house of Debre-Zeit Agricultural Research Center-Bishoftu.

The tested raw full-fat soybean (RFSB) was purchased from the local farmer, in Ethiopia. This test ingredient was then hammered to pass through a 0.2-mm sieve. Subsequently, 4 experimental diets were then formulated; containing such hammered RFFSB, which replaced the commercial SBM at 0, 15, 30 or 45% (equivalents to 0, 30, 60 or 90 g/kg of diet, respectively), which are also indicated as treatments: 1, 2, 3 and 4, respectively. While replacing the commercial SBM by RFFSB the major nutrient requirements were balanced (i.e., the "*iso-nitrogenous* and *iso-caloric" contents* of the diets, across the treatments were kept) by using other related ingredients, for example the meat meal. Feed-win (i.e., as a software) was used to formulate the experimental diets.

A total of 320 Bovan Brown female chicks (one-day-old) were purchased from Alema PLC Farm, Bishoftu-Ethiopia. Based on the requirements of these birds, common commercial diets (i.e., starter, grower and pullet diets) were freely/*adlib* offered. All required vaccinations were given. A 90 days trial was started when the age of the layers was 24 weeks, and then these birds were randomly allocated onto 4 experimental diets. Each experimental diet was also replicated 4 times and 17 laying birds per each replicate. Equal amounts of the experimental diets were weighed and then offered (depending on the number of birds per pen) throughout the trial period. Eggs were collected more than 3 times per day and then recorded.

$$\text{HDEP} = \frac{\text{Number of eggs collected per day}}{\text{Number of hens present at that day}} \times 100$$

HDEP: HDEP% (Hen-day egg production)

$$\text{AEM} = \text{PHDEP} \times \text{AEW}$$

AEM: Average egg mass (per hen per day in grams)
PHDEP: Per cent HDEP
AEW: Average egg weight in grams

$$\text{HHEP} = \frac{\text{NELP}}{NHLP}$$

NELP: Number of eggs laid in a period
NHLP: Number of hens present at beginning of laying period

$$HHEP = \frac{TNEDP}{TNHBP}$$

HHEP: HHEP (hen–housed egg production)
TNRFP: Total number of eggs laid during the period
TNHBP Total number of hens housed at the beginning of laying period.
The above formula were used based on TNAU (2023).

Egg Quality Evaluation

Forty-eight eggs, 12 per treatment (3 eggs per pen) were randomly selected on 30th day after the commencement of the trial. Other 2 samplings were also taken on the 60th and 90th days after the commencement of this trial. Totally, 144 eggs were sampled and evaluated for each of the following egg quality measuring parameters.

The external egg quality parameters were assessed in terms of egg weight and egg shape index. After breaking the egg, near to the sharpen end, and carefully separating and dropping the contents, internal egg quality measuring parameters were measured, in terms of shell weight, shell thickness, yolk weight, yolk height and yolk color. Shell thickness was measured by the digital caliper while removing the internal membranes. While measuring this thickness, the average value was taken from blunt, middle, and sharp points of the egg.

The yolk color was determined by comparing the color of a properly mixed yolk sample placed on a colorless glass with the color strips of Roche color fan measurement, which consists of 1 to 15 strips ranging from pale to orange-yellow. Shape index was computed using the following formula.

Egg weight was collected weekly by weighing all eggs per pen. Sensitive balance of 0.000lg to 20Kg capacity was used.

$$\text{Egg yolk index} = \frac{\text{Egg yolk height}}{\text{Yolk diameter}}$$

One per pen and 4 birds per treatment were randomly weighed and humanly slaughtered to evaluating the effects of partially replacing the commercial SBM by raw soybean on the development of internal organs. Some of the vital internal organs, such as pancreas, duodenum, intestines and gizzard were weighed and then evaluated as g/100 g of the corresponding sampled BWT of the bird.

Statistical Analysis

Descriptive statistics and ANNOVA were used to analyze the data using SPSS (IBM 26). The differences were considered to be significant at $p < 0.05$, and the significant differences between mean values were also separated using the Duncan's test.

One hen per pen, totally 4 birds per treatment were sampled and killed to evaluating the internal organ development. Intestine was measured with the contents. Commercial soybean meal (SBM) was replaced by raw, full-fat soybean at 0, 15, 30 or 45%, equivalent to 0, 30, 60 or 90 g/kg of the diets. NS= non-significant.

Results and Discussions

Chemical composition for the samples of raw soybean and commercial SBM are shown in Table 2. Maximum ether extract and apparent metabolizeable energy were recorded from samples of raw soybean as compared to the commercial SBM. The result showed that samples of raw soybean had more values of ether-extract/oil (14.7) and AME (12.6 MJ/kg) as compared to the commercial SBM (1.9 and 9.0), respectively. This current results agree with Erdaw (2016), who reported that full-fat soybean contains more (20.9%) fat (ether extracts) than other preparations, for example SBM.

Results due to the effects of the raw soybean supplementation, on the organ development of the layers are shown in Table 3. As shown in Table 3, replacing the commercial SBM by raw soybean (up to 45%) in the matured (i.e., starting from 24 weeks of age) layer diets had no negative effects in the final live BWT and also on the vital organ developments, such as pancreas, duodenum, intestine and gizzard.

These results are in line with Petričević et al. (2014), who reported that differences in body weights, food consumption, occurrence of defective eggs and the relative weight of the pancreas were not significantly influenced by the studied raw soybean or by their interaction effects.

The current results contradict with other research-scholars (Latshaw & Clayton, 1976; ASA, 2004; Erdaw and Beyene, 2018; Mammo et al., 2019), who reported that pancreas and duodenum weights increased at an increasing level of raw soybeans. The reason why these current results had no influences on the vital organ development might be due to the age of the layers (>24 weeks), which enabled them to resists the negative effects of anti-nutrients in the raw soybean.

Table 1. Ingredients (kg) and nutrient compositions

	T_1	T_2	T_3	T_4
Maize	65.5	64.5	65.6	63.5
Wheat middling	10	10	8.6	10
Soybean meal	20	17	14	11
Raw full-fat SB	0	3	6	9
Bone and meat meal	0	1	1.3	2
Limestone	2	2	2	2
Salt	0.4	0.4	0.4	0.4
Methionine	0.2	0.2	0.2	0.2
Lysine	0.4	0.4	0.4	0.4
Premix	0.5	0.5	0.5	0.5
Total	100	100	100	100
Major nutrient composition				
Crude protein (CP), %	16.154	16.105	16.129	16.285
Metabolizeable energy, in kcal/kg	2808.22	2813.255	2839.628	2834.405

Table 2. Chemical composition of raw full fat soybean and commercial SBM

Parameters	Raw full fat soybean	Commercial SBM
Dry matter	92.4	91.5
Crude proteins	38.2	42
Crude fiber	6.2	3.8
Ether extract	14.7	1.9
Calculated AME (MJ/kg)	12.6	9

Table 3. Effects of partially replacing the comm. SBM by raw soybean, in their diets on the organ development of sampled birds of layers (g/100g)

Treatments	BWT per bird	Intestines	Pancreas	Duodenum	Gizzard
1	1805	4.8	0.27	0.89	4.71
2	1767.5	4.4	0.25	1.02	4.68
3	1890	4.7	0.25	0.78	3.83
4	1897.5	4.5	0.26	0.85	4.19
SEM	0.4	1.6	18.36	0.21	0.01
Significance	NS	NS	NS	NS	Ns

Table 4. Effects of partially replacing commercial SBM by raw soybean, in layer-diets, on egg production and qualities

Treatments	BWT, g/ bird	HHEP, %	HDEP, %	Egg WT,g	Egg shape index	Egg shell thickness	Egg yolk index	Egg yolk colour	Egg shell WT, g
1	1605	78.5	80.7	61.5	77.5	0.37^{b}	0.45	1.29^{ab}	6.02^{b}
2	1567	73.1	75.8	63.2	75.2	0.42^{a}	0.47	1.20^{b}	6.83^{a}
3	1589	73.1	75.4	62.5	78.1	0.42^{a}	0.46	1.51^{ab}	6.65^{ab}
4	1565	72.7	75.4	61.7	76.2	0.39^{ab}	0.46	1.59^{a}	6.37^{ab}
SEM	55.9	5.9	1.6	0.4	1.0	0.19	0.01	0.05	0.10
Significance	NS	NS	NS	NS	NS	0.027	NS	0.009	0.029

BWT = body weights; HHEP = hen-housed egg production; HDSP = hen day egg production.; WT = weight; HHEP = hen-housed egg production; HDEP = hen-day egg production. Commercial soybean meal (SBM) was replaced by raw, full-fat soybean at 0, 15, 30 or 45%, equivalents to 0, 30, 60 or 90 g/kg of the diets. ^{a,b} means that the same superscripts in the columns are non-significant. NS = non-significant; SEM = standard error of mean

Findings on egg production and quality that were influenced by the effects of partially replacing the commercial SBM by raw soybean in layer-diets are shown in Table 4. Partially replacing the commercial SBM by raw soybean in diets of layers had no significant effects on average BWT, HHEP, HDEP and on the major egg quality measuring parameters, such as average egg WT, egg shape index and egg yolk index; however, there were significant differences on egg-shell Wt, egg-yolk color and egg shell thickness. These current results are against to that of Petričević et al. (2014), who reported that 8% of raw soybean supplementation, in the mixtures significantly reduced the number of eggs laid. The current result is also contradicting with Latshaw & Clayton (1976), who reported that only the hens fed on diets, containing 20% raw soybeans laid significantly fewer eggs than hens fed no raw soybeans.

Although the commercial SBM was replaced by the raw soybean at 45%-in the layer diets, egg production as well as egg quality was not significantly affected. The main reason for these findings might be due to the advancement of the age that enabled the layers to successfully resist the negative impacts of ANFs in the raw soybean. This thought is supported by ASA (2004) that reported as tolerance to the ANFs depends on the age of the birds.

Conclusion and Recommendations

Replacing the commercial SBM by RFSB (up to 45%) in diets of laying hens (mainly starting at 24 weeks of age) did not significantly influence both the egg production and egg qualities, as compared to the control group. It is therefore recommended to replace the commercial SBM by raw soybean, up to 45% in diets of laying hens. It is also specially recommended to replace the SBM by RFFSB in diets of laying hens when there is no accessibility/unavailable of the commercial SBM or when the SBM is relatively expensive.

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Evaluation of Consumer Attitudes Regarding Local Brand Milk and Dairy Products: Case of Süleymanpaşa Districts of Tekirdağ-Türkiye

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ABSTRACT

Since the concept of brand has a wide and diverse range, it is divided into different groups from different perspectives. Local brands are products that are produced, manufactured, and sold by a company in a specific city or that are distributed in a constrained area. This study's primary goal is to assess how local brands selling milk and dairy products in a particular region are perceived by local consumers. It also seeks to analyze the standing of local producers in this sector and consumer attitudes toward regionally branded goods by highlighting the significance of milk and dairy products in terms of human health. The study makes use of survey information from 381 households in Süleymanpaşa Districts of Tekirdağ-Türkiye. The data were analyzed using fundamental statistical techniques, factor analysis, and logistic regression analysis. In the survey, it was found that 85.0% of participants were familiar with the idea of local brand, while just 15.0% were not. Consumers who said they buy local brand milk and dairy products made up 78% of the sample. Consumers found local products to be more natural and tastier than national brands. According to the factor analysis, the judgements influencing customers' preference for local brand milk and dairy products were classified into five factors. These factors are named as naturalness and quality, price and promotion, health, food safety, brand and image. Logistic regression analysis was used to explain the association between purchasing local brand milk and dairy products and factor scores, as well as knowing the notion of local brand. Those who favor "Naturalness and Quality" in purchased milk and dairy products are nearly three times more likely than those who do not to purchase local brand milk and dairy goods. With a probability of 68.4%, those who do not understand the notion of local brand will not purchase local branded products.

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Introduction

Milk is one of the most important foods that humans must consume in order to achieve their nutritional needs. It serves as the first dietary supply for both humans and mammals following birth. Milk, which provides important animal protein, fat, lactose, vitamins, and minerals, is essential for adequate and well-balanced nutrition at all ages, from infancy to old life. Milk is a food that is easy to consume and digest. The production of milk and dairy products is critical to human health, and implementing food safety regulations in this process imposes significant responsibility on producers at both the national and local levels. Additionally, because milk contains several vitamins and minerals like calcium, proteins, vitamin A, and vitamin D, consumers view it as a significant food source, particularly when it comes to calcium for bone and dental health (Özsayın, 2017).

World milk production is projected to grow faster than other major agricultural products over the next decade, increasing by 1.7% annually to 1030 million tons by 2030 (OECD/FAO, 2021). Data from the FAO for 2022 indicate that 930 million tons of milk will be produced worldwide in that year. Cow's milk makes up about 81% of this production, while buffalo milk makes up 15.4%. The largest production shares are held by the USA (102.7 million tons), EU (159.3 million tons), and India (213.8 million tons) (FAOSTAT, 2022). Over the next decade, India and Pakistan are expected to contribute more than half of the growth in world milk production. It is anticipated that production in the European Union would expand more slowly than globally.

The amount of raw milk produced in Turkey in the same period was 21.5 million tons. In 2021, the raw milk production forecast was 23 million tons and decreased by

7.1% in 2022. The amount of milk produced is 92.3% cow's milk and 4.9% sheep's milk (TUIK, 2022). Approximately 15% of the cow's milk collected by commercial dairies in Turkey is delivered to consumers as drinking milk. The amount of drinking milk production in Turkey decreased in 2022 compared to the previous year (1.7%) and was realized as 1.5 million tons (TEPGE, 2023). According to 2021 data, total cheese production in Turkey was 763 thousand tons (Anonymous, 2022).

Milk consumption is considered as an indicator of development for countries. Milk and dairy product consumption values are quite high in countries with adequate and healthy nutrition (Onurlubaş and Çakırlar, 2016). Milk is a food that can be consumed directly, and there are also food such as cheese and yogurt produced from milk. Since milk has been a food since antiquity, every civilization has its unique customs for processing milk. Dairy products produced with traditional methods have enabled each society to have a unique palate and led to the emergence of products with very different flavors from milk.

Global milk and dairy product consumption is estimated to be 116.9 kg per person year based on 2020 data. Europe consumes the most milk per person year, whereas Africa consumes the least (Anonymous, 2022). As of 2022, cheese consumption was 20.9 kg per capita in the EU and 17.9 kg in the USA. Annual per capita butter consumption is highest in New Zealand (6.2 kg) and many Northern European countries (4.2 kg). In Turkey, products such as milk, yogurt, feta cheese and ayran are also more widely consumed, while drinking milk is generally consumed as whole milk (TEPGE, 2023). Due to the high level of informality in Turkey, the amount of milk consumption varies. According to the report prepared by the Chamber of Agricultural Engineers (2018), per capita drinking milk consumption was estimated at approximately 41.5 kg (ZMO, 2019; Engindeniz, et al. (2021). Originally, the term "trademark" refers to the designs, writings, and other features that were used to set one product or service apart from another. But today, the idea of a trademark is valued highly and is thought of from a much wider viewpoint. Today, brands are valued equally to the tangible assets of enterprises as an intangible asset. As the most important asset for businesses functioning on a worldwide scale, brands can serve a variety of purposes (Çakırer, 2013).

In today's competitive marketplace, a brand is more than just a name; it's also a commercial asset that provides a product identity and personality, affects consumer perceptions, and directs their purchasing decisions. Businesses and their customers can develop and shape relationships via the use of brands. Additionally, brands, which are now seen as part of a company's intellectual capital, have evolved into a commercial asset separate from their associated products. As a result, brand building for entrepreneurs necessitates a set of actions covering nearly every aspect of the company (Schultz and Barnes, 1999).

According to Philip Kotler (2000), as consumers become familiar and loyal to the brand, there is an opportunity to reduce the company's marketing expenditure in the long run. Increased demand for certain brands creates an advantage for the businesses, distributors and retailers that own that brand. When the brand is perceived to be of high quality, businesses may have the advantage of being able to sell that product at a higher price than competing products. The trust provided by the brand name allows manufacturers to easily expand their product lines.

As stated by İslamoğlu and Fırat (2011), a brand should provide the desired, desired and expected satisfaction by the consumer. At the same time, the brand should be able to cooperate with the consumer on satisfaction. A long-term relationship should be established between the consumer and a strong brand, based on trust, empathy and free from risks. Since the concept of brand is a multifaceted and broad concept, researchers have developed different categorization methods according to different perspectives. They have classified trademarks in various ways according to different criteria such as purpose of use, trademark right ownership, form, registration status, geographical area of operation (Şehirali, 1998; Özel, 2002; Eymen, 2007; Yarıcı, 2007).

Local brands, which are exclusive to a particular country or a limited geographic region, are said to help close the gap between the national economy and individual affluence (Natarajan & Thiripurasundari, 2010). Local consumer groups are the primary target of local brands' attention (Kotler and Armstrong, 2007). For regional companies that have had difficulty creating their own goods and identities, local methods provide a feasible alternative (Harun et al., 2010). Schuiling and Kapferer (2004), local brands have advantages such as the ability to respond more effectively to local needs, the ability to adopt flexible pricing approaches, the flexibility to adapt more easily to competitive conditions, the ability to manage the product range in a more adaptable way, and the ability to enter new markets faster. Local brands emphasize consumer awareness, trust and perception of quality rather than strong brand image compared to national and international brands. It is extremely tough to break customers' allegiance to heritage brands in industries where advertising is not as important. The foundation of local companies' competitive advantage in these markets is the trust relationships they have built with customers (Kapferer, 2002). Despite all these advantages, local brands also have some disadvantages. The small scale of the products sold by local brands increases production and marketing costs (Schuiling and Kapferer, 2004). Possible logistics problems that local brands may experience may cause them to fail to meet local requirements in different regions (Kotler and Armstrong, 2008).Numerous research, locally and nationally, have been carried out on the consumption of milk and dairy products as well as the variables influencing consumer preferences (Gül, 1987; Vural, 2001; Akbay and Tiryaki, 2007; Erdal and Tokgöz, 2011; Özcan, 2011; Karakaya and Akbay, 2014; Terin, 2014; Kızıloğlu, 2014; Yorgancılar, 2014; Terin et al., 2015; Yazıcı, 2016; Karakaya and Özkan, 2020; Engindeniz, et al., 2021; Yılmaz, et al., 2022). The common point of these studies is that socio-demographic and economic characteristics are effective in individuals' preferences for milk and dairy products. Edirisinghe and Athauda (2009) examined the relationships between demographic and socio-economic characteristics of Sri Lankan consumers in an ordered logistic regression and showed that age, cost and attitudes towards milk use and

nutritional attitudes were the main factors affecting milk consumption. In addition, household monthly income, health problems and education level were found to play a greater role in consumption.

In contrast to other studies, this study aims to evaluate consumer perceptions of local brands operating in a certain geographical area in the milk and dairy products market. Additionally, by emphasizing the importance of milk and dairy products in terms of human health, the point at which local milk and dairy producers stand on this issue and the attitudes of consumers towards local brand milk and dairy products were examined.

Material and Methods

The research material includes data obtained from face-to-face interviews with consumers residing in Süleymanpaşa district of Tekirdağ province and questionnaires presented to these individuals (Figure 1).

Figure 1. Research Area

The data of this study was obtained from the field study conducted in 2019 and does not require ethics committee approval. According to Turkish Statistical Institute (TUIK) data, there are 48,000 households in Süleymanpaşa district of Tekirdağ province. To determine the number of questionnaires representative of households, the "proportional sampling" method will be used, which is based on Yamane's (2009) formula. A 95% confidence interval and a 5% margin of error were included in equation 1. Furthermore, p=q=0.5 was chosen to achieve the maximum sample size because there hasn't been any research on the use of local brand milk and dairy products in the research region. (Equation 1).

$$n = \frac{N \times t^2 \times p \times q}{d^2 \times (N-1) + t^2 \times p \times q} \quad (1)$$

n: number of people to be surveyed, N: 48,000 (number of households in Tekirdağ Süleymanpaşa) (TUIK, 2020), p: 0.5 (those who buy local brand milk and dairy products), q: 0,5 (those who do not buy local brand milk and dairy products), t: 1.96 (value in t table according to 5% accepted margin of error), d: 0.05 (sampling error accepted according to frequency of occurrence)

As a sampling method, random sampling selection technique was used in Tekirdağ province. With this method, 381 different households were represented and face-to-face questionnaires were applied to consumers. Means, frequency distributions, factor analysis and logistic regression analysis were used to explain consumers' attitudes towards the purchase of branded milk and dairy products.

Factor analysis is a multivariate statistical method and its aim is to obtain a limited number of unrelated and meaningful new variables (factors or dimensions) by combining p interdependent variables. This method aims to discover the hidden structures underlying the factors. Factor analysis also involves the process of obtaining descriptions of common factors or new concepts, called factorization, using the factor loading values of items. This process aims to reveal functional explanations of concepts (Büyüköztürk, 2018).Factor analysis aims to reveal random factors reflecting the classification, which cannot be observed from the p variables in the data matrix x, which are observed and correlated between them, but which emerge when the variables come together. These new derived variables are called factors. It is used to reveal hidden dimensions that are known to exist but cannot be determined by direct observation. The most common use is to reduce and simplify much larger data sets (Karagöz, 1991).

Logistic regression analysis is a method used to model the relationship between one or more independent variables and the dependent variable. In this analysis, the dependent variable has a categorical characteristic, while the independent variable(s) can have both continuous and categorical characteristics. Binary logistic regression analysis refers to models where the dependent variable has only two categories. In this model, the occurrence and non-occurrence of the event are represented as 0 and 1. The ratio of the probability of the event occurring to the probability of the event not occurring is the "odds ratio" Odds ratio can take values between 0 and +∞ (Karcı and Arlı, 2018).

Results and Discussion

The study's participants were divided into 46.7% men and 53.3% women. When age categories are examined, it is discovered that 28.1% of participants are between the ages of 25 and 34, and around 37% are between the ages of 35 and 54. When the consumers' education level is examined, 26.3% are primary school graduates, 21.3% are secondary school graduates, 30.4% are high school graduates, 11.8% are undergraduate graduates, and 10.2% are postgraduate and above. In the study by Abdikoğlu et al (2018), the rate of high school graduates was found to be 27%, and 28.3% in Oraman, et al (2011). In Karakaya and Özkan's (2020) study analyzing the factors affecting the preferences of consumers in Antalya province for retailer branded milk and dairy products, the rate of postgraduate graduates was found to be 9.8%.

The average monthly household income of the surveyed consumers was found to be 3,761.02 TL (639.63 $). In addition, the average monthly food expenditure of the household was calculated as 1,274.39 TL (216.73 $). Consumers allocate approximately 33.9% of their income to food expenditures. Akbay and Tiryaki (2007), in their study in Kahramanmaraş province, found that the share of monthly food expenditures of consumers in their total income was 32.69%. Erdal and Tokgöz (2011), in their study on the consumption preferences of consumers for packaged and open milk, found that the ratio of food expenditures was approximately 17% of total income and 24% of total expenditures. When the monthly income distribution is analyzed, there is a concentration in the

range of 2,000 - 3,999 TL (340.12-680.10) and 4,000 - 5,999 TL (680.27-1020.23). Similarly, monthly food expenditures of households are concentrated in the range of 500 - 1,249 TL (85.03$- 212.41$) (Table 1). Based on demographic features, a broad consumer profile was developed, claims Vural (2001). The study found that the purchase behavior of milk and dairy products is influenced by consumer income. It was also discussed how education influences the decision-making process in a way that leads to collaborative decision-making.

Table 2 presents the prioritization of consumers' dairy purchasing locations and preferences based on the weighted scoring calculation. According to the findings, consumers primarily prefer supermarkets to purchase milk and dairy products. Notably, no consumers purchase these products from local markets or online sources. The data also reveal that after supermarkets, consumers tend to prefer to buy milk and dairy products directly from producers. Niyaz and Inan (2016) stated that 53% of the consumers in TR22 South Marmara Region prefer to buy milk and dairy products from the market and 36.8% prefer to buy directly from the producer. According to Engindeniz, et al. (2021), 80% of consumers buy milk and dairy products from the market, while the second place of purchase was producers. Similarly, Çelik, et al. (2005), in their study in Şanlıurfa province, found that 61.4% of consumers prefer to buy milk from supermarkets.

The average purchase amounts of milk and dairy products of consumers are given in Table 3. Accordingly, on average, households consume 9.48 liters of milk, 4.02 kg of feta cheese and 9.82 kg of yogurt per month. When calculated as annual consumption amounts, milk consumption was found to be 113.76 kg, cheese 48.24 kg and yogurt 117.84 kg per household. In the study, the average household size was determined as 3 people. Accordingly, per capita milk consumption in Süleymanpaşa district of Tekirdağ province was found to be 37.92 kg, cheese consumption 16.08 kg and yogurt consumption 39.28 kg per year. According to the report prepared by the National Milk Council in 2022, per capita drinking milk consumption was estimated as 39.1 kg, cheese consumption as 19.6 kg and yogurt consumption as 29 kg (Anonymous, 2022). In a study conducted in İzmir/Bornova district, it was determined that the annual milk consumption per capita was 37.43 kg, yogurt consumption was 32.84 kg and cheese consumption was 18.48 kg (Engindeniz, et al. 2021). In a study conducted in 2001 in Antalya province, milk consumption per capita was 15.3 kg/year and yogurt intake was 23.2 kg/year (Vural, 2001). In this regard, the amount of milk and dairy products consumed in the research region is comparable to previous studies.

Among the consumers who participated in the survey, 85.0% stated that they knew the concept of local brand and 15.0% stated that they did not know the concept of local brand (Table 4). 78% of the surveyed consumers stated that they purchased milk and dairy products with local brands.

Table 1. Demographic Characteristics of Consumers

	Oran (%)
Gender	
Male	46.7
Female	53.3
Age Groups	
25 - 34	28.1
35 - 44	17.2
45 - 54	20.7
55 - 64	22.4
65 years and older	11.6
Education Status	
Primary School	26.3
Middle School	21.3
High School	30.4
Undergraduate	11.8
Master's Degree and Above	10.2
Household Income Level	
2000 TL below (below 340.13$)	12.9
2.000 TL - 3.999 TL (340.13$ - 680.10$)	38.3
4.000 TL - 5.999 TL (680.27$ - 1020.24$)	37.8
6.000 TL - 7.999 TL (1020.40$ - 1360.37$)	9.4
8.000 TL and above (1360.54$ above)	1.6
Household Monthly Food Expenditure	
500 TL below (85.03$ below)	2.1
500 TL - 1.249 TL (85.03$ – 212.41$)	51.5
1.250 TL - 1.999 TL (212.58$ – 339.96$)	31.4
2.000 TL - 2.749 TL (340.13$ – 467.52$)	12.0
2.750 TL and above (467.69$ above)	3.0
Total	100.0

Table 2. Consumers' Preference Priorities for Dairy Products Purchase Location

	Super Market	Grocery	Delicatessen	Internet	Public/Street Bazaar /Farmers
Milk	1	3	-	-	2
Feta Cheese	1		2		3
Fresh Kashar Cheese	1	3	2		
Aged Kashar Cheese	1	2	3		
Yogurt	1	3			2
Butter	1		3		2
Cream	1	3			2

Table 3. Average Purchases of Milk and Dairy Products by Consumers

Milk and Dairy Products	Amount Consumed (kg or lt) (monthly)
Milk	9.488 lt
Feta Cheese	4.021 kg
Fresh Kashar Cheese	1.734 kg
Aged Kashar Cheese	1.698 lt
Yogurt	9.815 kg
Butter	1.340 kg
Cream	0.846 kg

Table 4. Consumers' Knowledge and Purchase of Local Brand Concept

Knowing the Concept of Local Brand	%
Knows	85.0
Doesn't know	15.0
Total	100.0
Consumers' Purchase Status of Local brand Milk and Dairy ProductsLocal brand	%
Purchasing	78.0
Not Purchasing	22.0
Total	100.0

Table 5. Consumers' Reasons for Not Purchasing Local brand Milk and Dairy Products

Reasons for Not Purchasing Local brand Milk and Dairy Products	%
Packaging	8.9
Storage Life	30.4
Price	19.0
Food Safety	18.5
Nutritional Values	6.0
Availability	17.3
Total	100.0

Among consumers who do not buy local brand milk and dairy products, 8.9% cited the packaging of the product as a problem, while 30.4% cited short storage time as a concern. In addition, 19.0% cited the price of the product, 18.5% were concerned about food safety, 6% were concerned about the adequacy of nutritional value, and 17.3% cited the ubiquitous availability of the products as a reason for not purchasing (Table 5). In İzmir province, Kahraman (2016) conducted a study on brand selectivity in milk consumption habits and discovered that characteristics including trust, health, quality, taste, and flavor were significant determinants of both brand preference and consumption patterns.

In Table 6, the relationship between the demographic structure of consumers and their purchasing status of local brand milk and dairy products is analyzed. Accordingly, there is no relationship between being under and over 35 years of age and purchasing status ($p>0.05$). There is a significant relationship between education level and purchasing status ($p<0.05$). According to Cramer's V coefficient, there is a weak relationship. The Cramer V coefficient provides information about the strength of the relationship between two categorical variables (Öztuna et al., 2008). This coefficient is between 0 and 1 (Healey, 2011). The purchasing rate is 71% for primary school graduates, 79% for secondary school graduates, 82.6% for high school graduates, 66.7% for undergraduate graduates and 92.3% for graduate graduates. There is also a relationship between purchase status and consumer income ($p<0.01$). While 66.7% of consumers with an income of 2000 TL (340.13$) or less buy local brand milk and dairy products, 88.1% of consumers with 4000 TL (680.27$) and above purchase. While local brand milk and dairy products are purchased by 74.7% of male customers, 80.7% of female consumers do the same. According to Karakaya and Özkan (2020), gender influences the chance of eating retailer-branded milk and dairy goods. Specifically, female consumers are 5.84 times more likely than male consumers to consume retailer-branded milk and dairy products.

Factor analysis was used to assess consumer perceptions of the attributes, marketing strategy, and food safety of regionally branded milk and dairy products. Table 7 provides details on the variables utilized in factor analysis. In this context, the majority of consumers (85.04%) stated that they find local brand milk and dairy products more natural compared to national brands. The idea that local brands have a shorter storage life because they are natural is also noteworthy at 72.71%. At the same time, consumers believe that local brand milk products preserve traditional flavors. This reflects the role of consumers prefer to buy local brand milk and dairy products to support local producers, demonstrating a desire to strengthen the local economy (63.26%).In terms of price, 66.93% of consumers think that the price of local brand milk and dairy products is in line with their quality. This suggests that local brands are also economically attractive to consumers by offering affordable alternatives. Confidence in the accuracy of label information and perceptions of the absence of harmful substances in the product content support consumers' positive attitudes towards local brands in preserving cultural values and long-established flavors. Customers have noticed that there is not enough marketing or advertising for regionally branded milk and dairy products on the market. 65.09% of customers said there weren't enough promotional activities, while 61.94% said the advertising weren't good enough.

In Table 9, the reliability of the scale created for the judgments presented in the questionnaire was measured by Cronbach's Alpha method. The calculated value of 0.826 shows that the data is suitable for analysis. The suitability of the obtained data for factor analysis was tested with KMO (sample equivalence test) and Bartlett's (sphericity test). The KMO value was found to be 0.795. The fact that this value is greater than 0.50 indicates that the data are suitable for factor analysis (Table 10).

In the total variance explained, five factors explained 65.005% of the total variance, with factor I explains for 29.428% of the total variance, factor II explains for 15.337%, factor III explains for 7.181%, factor IV explains for 6.645%, and factor V explains for 6.414% (Table 10).

According to the results of the applied factor analysis, the judgments affecting consumers' preference for local brand milk and dairy products can be analyzed under five different factors (Table 11). Factor I is named as "Naturalness and Quality". In this factor, the judgments that affect consumers' preference for local brand products are summarized as the price of the products is affordable according to the quality of the products, traditional flavors are preserved and the storage life of local brands is short because they are natural. Factor II is named as "Price and Promotion". Under this factor, factors such as availability of local brand products, promotions, product campaigns and lack of variety are included.

Consumers prefer local brand products because they are more affordable and they can be found everywhere. Factor III is named as "Health". In this factor, the judgments that affect consumers' preference for local brand products include that the products are produced in accordance with hygiene conditions, the packaging is healthy and hygienic, and the producers are well controlled by supervisory institutions. Factor IV is named as "Food Safety". Under this factor, the judgments that affect consumers' preference for local brand products include the absence of harmful additives in the product content and that local brands comply with food safety standards. Factor V is named as "Brand and image". Under this factor, the reasons why consumers prefer local brands include wanting to support local producers and finding local brands' products more delicious (Table 11).

A binary logistic regression analysis was performed to identify the variables influencing customers' decisions to buy local brand dairy and milk products. In the logistic regression model, the binary categorical variable "Consumers' Purchase Status of Local brand Milk and Dairy Products" (1: Purchases, 0: Does not purchase) was used as the dependent variable. As independent variables, the factor scores obtained from factor analysis ("Naturalness and Quality", "Price and Promotion", "Health", "Food Safety", "Brand and Image") and "Knowing the Concept of Local Brand (1: Knows, 0: Does Not Know)" are included in the model. Descriptive statistics for these variables are given in Table 12.

Table 6. Relationship between Demographic Structure and Local brand Milk-Dairy Product Purchase

	Purchasing (%) (freq.)	Does not purchase (%) (freq.)	
Age			
Under 35 years of age	79.4(85)	20.6(22)	χ^2 : 0.191 p:0.662
35 and above	77.4(212)	22.6(62)	Cramer's V : 0.0224
Education			
Primary School	71.0(71)	29.0(29)	χ^2 : 15.1 p:0.015** Cramer's V : 0.18
Middle School	79.0(64)	21.0(17)	
High School	82.6(95)	17.4(20)	
Undergraduate	66.7(30)	33.3(15)	
Master's Degree and Higher	92.3(36)	7.7(3)	
Gender			
Male	74.7(133)	25.3(45)	χ^2 : 1.96 p:0.16
Female	80.7(163)	19.3(39)	Cramer's V : 0.0718
Income			
2000 and below	66.7 (60)	33.3 (30)	χ^2 : 15.1 p:0.001* Cramer's V : 0.201
2001-4000 TL (340.30$ - 680.27$)	75.7 (112)	24.3 (36)	
4000 above (680.27$ above)	88.1 (118)	11.9 (16)	

*p<0.01, **p<0.05

Table 7. Descriptive Statistics of Variables Used in Factor Analysis

	Frequency Distribution (%)					Mean	Std.Dev
	1	2	3	4	5		
I find the price of local brand milk and dairy products affordable according to their quality (K115).	10.5	7.87	14.7	38.58	28.35	3.66	1.26
I believe that local brand milk and dairy products preserve traditional flavors (K113)	8.14	6.82	14.44	38.58	32.02	3.80	1.20
I think local brand milk and dairy products have a shorter storage life because they are natural.(K114)	5.77	8.92	12.6	34.91	37.8	3.90	1.17
I find local brand milk and dairy products more natural (K103)	3.41	3.67	7.87	43.31	41.73	4.16	0.96
Local milk and dairy products have more promotions / product campaigns (K101)	35.17	29.92	10.24	15.22	9.45	2.34	1.34
I find advertisements and promotions of local brand milk and dairy products sufficient (K98)	33.33	28.61	19.95	8.66	9.45	2.32	1.28
I can find local brand milk and dairy products everywhere (K99)	16.05	17.11	22.63	26.32	17.89	3.13	1.33
I find the product diversity of local brand milk and dairy products sufficient. (K100)	21.0	22.83	18.37	23.1	14.7	2.88	1.37
I prefer local brand milk and dairy products because they are more affordable. (K97)	22.57	19.16	18.11	22.83	17.32	2.93	1.42
I think that local brand milk and dairy products produce in accordance with hygiene conditions. (K104)	4.46	9.19	29.13	34.65	22.57	3.62	1.07
The packaging of local brand milk and dairy products is healthy and hygienic. (K106)	6.3	5.77	29.66	37.01	21.26	3.61	1.08
I think that companies producing local brand milk and dairy products are controlled well enough by supervisory institutions (K109)	17.06	19.69	28.08	22.05	13.12	2.94	1.28
I think that local brand milk and dairy products do not contain additives that are harmful to health.(K108)	14.17	13.12	11.55	36.22	24.93	3.45	1.37
I think local brand milk and dairy products fully comply with food safety standards (K110)	12.86	17.32	29.4	24.67	15.75	3.13	1.25
I try to buy local brand milk and dairy products to support local producers. (K112)	7.61	9.97	19.16	31.76	31.5	3.70	1.23
I find the products produced by local brand milk and dairy products more delicious (K102)	5.51	7.61	13.39	30.45	43.04	3.98	1.17

1: Strongly disagree, 2: Disagree, 3: Neutral, 4: Agree, 5: Strongly agree

Table 8. Reliability Analysis

Cronbach's Alpha	Cronbach's Alpha Based on Standardized Items	N of Items
.826	.830	16

Table 9. Test of Sample Equivalence (KMO) and Test of Sphericity (Bartlett)

Kaiser-Meyer-Olkin Measurement of Sampling Adequacy		.795
Bartlett's Test of Sphericity	Approximate Chi-Square	1998.460
	df	120
	Sig.	.000

Table 10. Total Variance Explained

C	Initial Eigenvalues			Square Loadings Extraction Sums			Square Loadings Rotation Totals		
	Total	Variance (%)	Cumulative %	Total	Variance %	Cumulative %	Total	Variance %	Cumulative %
1	4.708	29.428	29.428	4.708	29.428	29.428	2.652	16.574	16.574
2	2.454	15.337	44.764	2.454	15.337	44.764	2.572	16.072	32.646
3	1.149	7.181	51.945	1.149	7.181	51.945	1.904	11.901	44.547
4	1.063	6.645	58.590	1.063	6.645	58.590	1.732	10.828	55.375
5	1.026	6.414	65.005	1.026	6.414	65.005	1.541	9.630	65.005
6	.753	4.707	69.712						
7	.741	4.632	74.344						
8	.622	3.889	78.233						
9	.614	3.838	82.071						
10	.565	3.533	85.604						
11	.466	2.915	88.519						
12	.461	2.880	91.399						
13	.450	2.815	94.213						
14	.384	2.402	96.616						
15	.302	1.885	98.501						
16	.240	1.499	100.000						

C: Component

Table 11. Rotated Component Matrix

		Components				
		1	2	3	4	5
Naturalness and Quality	I find the price of local brand milk and dairy products affordable according to their quality (K115).	.804				
	I believe that local brand milk and dairy products preserve traditional flavors (K113)	.736				
	I think local brand milk and dairy products have a shorter storage life because they are natural.(K114)	.683				
	I find local brand milk and dairy products more natural (K103)	.662				
Price and Promotion	Local brand milk and dairy products have more promotions / product campaigns (K101)		.794			
	I find advertisements and promotions of local brand milk and dairy products sufficient (K98)		.777			
	I can find local brand milk and dairy products everywhere (K99)		.678			
	I find the product diversity of local brand milk and dairy products sufficient. (K100)		.654			
	I prefer local brand milk and dairy products because they are more affordable. (K97)		.507			
Health	I think that local brand milk and dairy products produce in accordance with hygiene conditions. (K104)			.780		
	The packaging of local brand milk and dairy products is healthy and hygienic. (K106)			.669		
	I think that companies producing local brand milk and dairy products are controlled well enough by supervisory institutions (K109)			.590		
Food Safety	I think that local brand milk and dairy products do not contain additives that are harmful to health.(K108)				.755	
	I think local brand milk and dairy products fully comply with food safety standards (K110)				.743	
Brand and image	I try to buy local brand milk and dairy products to support local producers. (K112)					.686
	I find the products produced by local brand milk and dairy products more delicious (K102)					.628

Table 12. Descriptive Statistics of Variables

	Mean	Std.Dev
Consumers' Purchase Status of Local brand Milk and Dairy Products (1: Purchases) (Dependent Variable)	0.78	0.42
Naturalness and Quality	3.88	0.91
Price and Promotion	2.72	0.96
Health	3.39	0.89
Food Safety	3.29	1.12
Brand and image	3.84	0.96
Knowing the Concept of Local Brand (1: Knows the concept of local brand)	0.85	0.36

The averages for the factors were calculated from the averages of the answers given to the judgments included in each factor (1: Strongly disagree, 2: Disagree, 3: Neutral, 4: Agree, 5: Strongly agree). It is assumed that factors with a mean of up to 2.5 are perceived by consumers as strongly negative, factors with a mean between 2.6-2.9 are perceived by consumers as relatively negative, factors with a mean between 3.0-3.4 are perceived by consumers as relatively positive and factors with a mean above 3.5 are perceived by consumers as strongly positive. In light of this, it is noted that customers' perceptions of the "Naturalness and Quality" of local brand milk and dairy products are favorable in comparison to those of other brands (3.88). Similarly, customers gave local brand milk and dairy products a favorable evaluation in terms of their "Brand and image" (3.84). When analyzing the logistic regression findings, this was taken into account.

First, the test results showing the overall fit of the model are presented in Table 13. It is seen that the overall significance of the model, i.e. the goodness of fit, is statistically significant ($p<0.01$).

According to the Hosmer and Lemeshow test result, the estimated logistic regression model was found to be appropriate for the data ($p=0.383$) (Table 14).Cox & Snell R^2 and Nagelkerke R^2 values indicate the magnitude of the variance explained by the model in the dependent variable. The overall fit of the model was found to be good (Cox & Snell $R^2=0.243$; Nagelkerke $R^2=0.373$). Accordingly, 37% of the local brand purchase status is explained by the independent variables (Table 15). Coefficient estimates and odds ratios of binary logistic regression analysis are presented in Table 16.

Customers who positively view local brand milk and dairy products in terms of "Naturalness and Quality" are

three times more likely to buy them than consumers who do not, as demonstrated by the coefficients in Table 16. In other words, consumers who perceive local brand milk and dairy products as more natural and of higher quality than other brands are more likely to purchase these products. Furthermore, consumers are 1.6 times more likely to buy local brand milk and dairy products than those who have a positive view of the "Brand and image" aspect. As can be seen from Table 11, the "Brand and image" factor includes judgments about supporting local producers and the brand image of local brand milk and dairy products. Consumers' perceptions of the "Food safety" factor are relatively positive (3.29). However, 29.4% of the consumers are indifferent to the statement "I think local brands fully comply with food safety standards". In other words, consumers are cautious about local brand milk and dairy products in terms of food safety. Therefore, consumers who pay attention to the "Food Safety" factor are 1.16 times less likely to purchase local brand milk and dairy products than others (1/0.862=1.16). local brand In the case of knowing the concept of local brand, when the reference category is taken as "Knows", those who do not know this concept will not purchase local brand products with a probability of 68.4% ((1-0.316)*100 = 68.4%).

Table 13. General Test of Model Coefficients

	Chi-square	df	Sig.
Step	105.908	6	.000
Block	105.908	6	.000
Model	105.908	6	.000

Table 14. Hosmer and Lemeshow Test

Step	Chi-square	df	Sig.
1	8.534	8	.383

Table 15. Model Summary

Step	-2 Log likelihood	Cox & Snell R^2	Nagelkerke R^2
1	295.552a	.243	.373

Table 16. Model Estimation Results

	β	S.E.	Wald	df	Sig.	Exp(β)(odds)
Naturalness and Quality	1.107	.160	48.023	1	.000	3.027
Price and Promotion	.150	.159	.888	1	.346	1.162
Health	-.057	.153	.141	1	.707	.944
Food Safety	-.149	.162	.844	1	.358	.862
Brand and image	.459	.142	10.390	1	.001	1.583
Knowing the Concept of Local Brand[1]	-1.154	.369	9.756	1	.002	.316
Constant	1.806	.179	101.412	1	.000	6.086

Refereance category : [1]Knows

In conclusion, local brand milk and dairy products attract significant interest among consumers and are evaluated with various positive attitudes. Factors such as naturalness, taste, health, affordable prices and trust in the product production processes offer great potential for local brand producers to increase customer satisfaction and build a wider customer base. Consumers' positive attitudes towards product production processes are also evident. Consumers have a positive view that local brand milk and dairy products comply with the storage standards from the raw material from the producer to the product, that production is carried out in accordance with hygiene conditions, and that product packaging is healthy and hygienic.

Studies conducted on this subject explain that hygiene conditions in milk and milk products and the idea that the product is healthy are important factors for the purchase of the product, and that households pay the most attention to the expiration date of the product during purchase (Gündüz, et al., 2013). It is important in terms of guiding consumer behavior and awareness in accessing healthy food with the studies to be conducted and guiding the decisions to be taken. For this purpose, it is very important to popularize healthy milk processing techniques and to provide necessary trainings to producers (Yılmaz, et al., 2022). Enterprises producing local brand milk and dairy products need to switch to production processes that will gain the trust of consumers and comply with food safety criteria. According to the results obtained in the research, it was seen that the most important criterion for consumers when purchasing milk and dairy products is that the product is healthy. Therefore, local producers should manage the processes of milk from the producer to the finished product in accordance with human health and operate in accordance with food safety systems related to milk production.

It should be considered as a great advantage for local brand producers that consumers know the concept of local brand and prefer local brand milk and dairy products to national brands when they consider criteria such as naturalness, price, health and taste. In the study, it was determined that consumers mostly buy milk and dairy products from supermarkets. In addition, it was observed that they prefer to buy directly from the producer after the supermarket. This shows that the urban population prefers to obtain milk and dairy products from supermarkets. At the same time, the fact that consumers prefer to buy milk and dairy products directly from the producer after supermarkets may indicate that street dairying still continues.

In order to increase their sales, enterprises operating in the milk and dairy products sector should produce products and services in line with their wishes and expectations by bringing consumer preferences to the forefront (Onurlubaş & Çakırlar, 2016). Local brands have the opportunity to establish a trust relationship directly with consumers and can meet their needs directly. They can also be more flexible on price than national brands. Local brand product producers must first show and emphasize to consumers that they preserve traditional flavors and establish a trusted relationship. Global brands that establish close relationships with consumers can also be accepted as local brands by consumers after a certain period of time in local geographies where they have been operating for many years.

Consumers stated that they find the promotions, product variety and advertisements of local brand products inadequate. Local producers should develop advertising strategies that are compatible with today's technologies by using the advantages of the local brand. These advertisements should emphasize how to respond to consumers in the best and fastest way. At the same time, they should continue their efforts to increase the amount of shopping by creating a variety of products suitable for consumers' desires. By organizing campaigns, they can increase their sales and build a closer relationship with consumers. As a factor to increase consumer satisfaction, Topçu, Baran, and Denizli (2016) recommend implementing marketing tactics and strategies for the short supply chain, including the choice for low-income consumers to buy generic branded milk with high sensory quality qualities either directly from the farmer or from local producer milk processing facilities. To guarantee the long-term survival and sustainable existence of local producers who take use of their relative pricing advantage, this is a priority that must be given significant attention.

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Comparison of Extraction Techniques for Determining Bioactive Compounds and Antioxidant Activity of *Spirulina platensis*

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ABSTRACT

Spirulina platensis (S. platensis) is a high-nutrient blue-green algae that has been used as a food supplement for a long time. It contains carbohydrates, lipids, proteins, vitamins, minerals, and bioactive compounds essential for basic human nutrition. It is known to have anti-cancer, antioxidant, anti-inflammatory, neuroprotective, hepatoprotective, and hypocholesterolemic properties due to the bioactive compounds it contains. In this study, the effects of freeze-thawing, a rapid freezing (-20°C) and thawing (4°C) process, and ultrasonically assisted extraction techniques on the color, antioxidant capacity, total phenolic content, and phenolic composition of *Spirulina platensis* extracts were investigated. The antioxidant capacity of the extracts obtained was determined by two different methods, DPPH (2,2-diphenyl-1-picryl hydrazyl) and ABTS (2,2-azinobis (3-ethylbenzothiazollin-6-sulfonic acid)). The sugar profile was determined by HPLC-RID and phenolic composition was determined by HPLC-ESI-DAD-MS/MS. The antioxidant activity and total phenolic content of samples prepared by the freeze-thawing were higher than those prepared by ultrasonic-assisted conventional extraction technique. In addition to ferulic acid 4-O-glucuronide and brevifolin carboxylate, an isocoumarin derivative, as the dominant phenolic compound in *S. platensis* extracts, a total of 10 phenolic compounds including catechin isomer, resveratrol C-hexoside, myricetin, ferulic acid, gallic acid, phloroglucinol, and lutein were detected. Glucose was the predominant sugar in both samples. The total sugar content was higher in the freeze-thawed samples (217.92 mg/100 g DW) than in the ultrasonic-assisted conventional extraction technique (182.91 mg/100 g DW). *S. platensis* has a significant amount of antioxidants, valuable secondary metabolites, and potential commercial applications and medicinal properties, but releasing these compounds is difficult due to the cell wall. This study was carried out to determine how different extraction techniques alter the release of bioactive compounds.

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Introduction

Microalgae are seen as one of the sources with the highest biological activity in nature and contain many bioactive compounds in their structure (Alajil Alslibi, 2019). *Spirulina platensis* is a single-celled, filamentous, prokaryotic microalgae that thrives natively in alkaline waters of warm lakes and consists of about 15 species (Maddina et al., 2016). *S. platensis*, also known as *Arthrospira platensis*, is a protein-rich algae that the Aztecs historically collected from Lake Texcoco in Mexico (Saranraj & Sivasakthi 2014). This particular plant-based protein is unique in terms of its protein content. It contains all essential amino acids, including leucine, valine, isoleucine, tryptophan, methionine, phenylalanine, theanine, and lysine, as well as non-essential amino acids such as glycine, proline, arginine, cysteine, tyrosine, glutamine, alanine, serine, glutamate, and aspartate. It has a protein content of approximately 55-70% and is often referred to as a "superfood" since of provides abundant nutritional content (Maddina et al., 2016; Saranraj & Sivasakthi 2014).

S. platensis contains bioactive compounds, especially carotenoids, responsible for antioxidant activities, phycocyanin, an active protein, and phenolic acids (Maddiboyina et al., 2023). Phycocyanin acts as antioxidants that scavenge free radicals and prevent oxidative stress. These antioxidants can transform into pro-oxidants and protect the body against oxidative stress (Maddiboyina et al., 2023). Phenolic compounds are

naturally occurring antioxidants that are important sources of polyphenols synthesized by microalgae. These compounds contain one or more hydroxyl groups directly attached to the aromatic ring. According to studies by Ferreres et al. (2012) and Heffernan et al. (2015), polyphenols are widely recognized as crucially important chemical compounds.

S. platensis is easy for humans to digest as its cell wall consists of 86% digestible polysaccharides without hard cellulose. However, breaking down the cell wall with conventional extraction techniques is very difficult. Therefore, it is necessary to hydrolyze the covalent bonds in the structure to release phenolics or to disrupt the cell wall matrix (Arranz et al., 2010; Shahidi & Yeo, 2016). It is very important to use green extraction processes to increase efficiency, reduce processing time, and save energy. Due to its ability to lower process temperature, duration, and solvent consumption, ultrasound-assisted extraction (UAE) is gaining popularity as a substitute method (Ahmed et al., 2022). This study used freeze-thawing, which has been widely used in recent studies, in addition to ultrasonic-assisted conventional extraction (UACE). In UACE, ultrasonic waves disrupt the cell wall and accelerate mass transfer, enabling the desired bioactive components to be obtained in a shorter time and yield higher than classical techniques (Purdi et al., 2023). When a sound wave is intense, it can create empty spaces in a liquid by overcoming the gravitational forces that cause the liquid to relax. These empty spaces are known as 'cavitation bubbles' and grow larger with each sound wave cycle until they reach a critical size. Bubbles reaching the critical size collapse violently, and as a result of this collapse, the increase in temperature and pressure in the environment leads to the formation of microjets. Microjets cause surface peeling, erosion, cell wall destruction, and leakage of cell contents, thus enabling the extraction of natural compounds from various sources (Purdi et al., 2023). The freeze-thawing technique consists of rapid freezing at -20 °C and thawing at 4°C. This lysis method causes the cells to swell and eventually break by forming intracellular ice crystals during freezing and shrinking during thawing. Multiple cycles are required for effective lysis, and the process can be pretty long (Tan et al., 2020). This study investigated the effect of ultrasonically assisted conventional extraction and freeze/thaw technique on antioxidant activity, total phenolic content, sugar, and phenolic composition of *S. platensis* extracts. The antioxidant activity of the extracts was determined by 2 different methods, DPPH and ABTS, sugar profile by HPLC/RID, and phenolic composition by HPLC-MS/MS.

Material and Method

Chemicals

Folin-Ciocalteu reagent was obtained from Merck (Darmstadt, Germany), Trolox and 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazollin-6-sulfonic acid) (ABTS), phenolic compounds and sugar standards (sucrose, glucose, and fructose) were obtained from Sigma-Aldrich Chemical Co (St. Louis, USA). All solvents and chemicals used in this study were of chromatographic and analytical purity and were prepared daily.

Preparation of S. platensis Extracts

Commercial *Spirulina* samples were obtained as powder from a local market in Adana. Four grams of samples were weighed into a 100 ml Erlenmeyer flask, 100 ml of water was added and mixed for 15 minutes in a magnetic stirrer. The samples were kept in an ultrasonic water bath with an ultrasonic power of 60 W and a frequency of 40 kHz for 1 hour at 25 °Cice was added to prevent temperature rise, and then one of the samples was stirred overnight at room temperature in a magnetic stirrer, and the other was subjected to a rapid freezing (-20°C) and thawing (4°C) process repeated 4 times. At the end of the period, both samples were centrifuged at 7000 rpm for 15 minutes at 4°C. The resulting extracts were filtered through a 0.45 µm membrane filter and stored in a refrigerator at 4°C until analysis.

Antioxidant Capacity and Total Phenolic Content

DPPH method: DPPH (2,2, diphenyl 1-picry hydrazyl), which can measure the ability to inhibit free radicals, was used and the absorbance values were recorded in a UV-Vis spectrophotometer (BMG LABTECH, SPECTROstar Nano, Ortenberg, Germany) according to the results of measuring the change of the reaction in methanol against time (Brand-Williams et al., 1995; Kelebek et al., 2013). Briefly, 100 µl of each extract was mixed with 3.9 ml of DPPH solution and incubated in the dark at room temperature for 1 hour. The absorbance was then measured at 515 nm with a UV-visible spectrophotometer and the results are given as µmol Trolox/100g DW. *ABTS method:* It was performed according to the method of Saafi et al. (2009). To perform this method, mix 7 mM of ABTS (2,2'-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid) with 2.45 mM of potassium sulfate and keep it in the dark for around 12 to 16 hours. Then, dilute this solution with sodium acetate buffer (pH 4.5) to an absorbance of 0.700±0.01 at a wavelength of 734 nm in a spectrophotometer. After that, add 2.98 mL of the prepared buffer to 20 µL of the sample extract, wait for 10 minutes, and measure the absorbance in a UV-Vis spectrophotometer (BMG LABTECH, SPECTROstar Nano, Ortenberg, Germany) at a wavelength of 734 nm. The absorbance values obtained should be calculated by using a trolox (10-100 µmol/L) standard slope chart, and the results were expressed in µmol Trolox/ 100g.

Determination of total phenolic content: The basic principle of the analysis is based on the oxidation of phenolic compounds by reduction of the Folin-Ciocalteu reagent in a basic medium. To perform a spectrophotometric analysis, 200 µl of either extract or standard solution was added into a cuvette. Then, 1.5 ml of Folin-Ciocalteu reagent (diluted at a 1:10 ratio) was added to the cuvette and left for five minutes. Following that, 1.5 ml of 6% sodium carbonate solution was added to the cuvette. The mixture was then kept at room temperature and in the dark for 90 minutes. With increasing phenolic content, the blue-colored solution becomes darker. Absorbance differences were measured at 765 nm on a UV-Vis Spectrophotometer (BMG LABTECH, SPECTROstar Nano, Ortenberg, Germany). A 500 ppm solution of gallic acid was prepared for the calibration curve. Phenolic content was calculated based on the slope obtained from the calibration curve (Shahidi, 2015) and the results were expressed in mg GAE/ 100g.

Determination of Phenolic Compounds by HPLC-ESI-MS/MS

An Agilent Technologies HPLC system (model 1100) controlled by ChemStation software, was used to analyze phenolic compounds. The HPLC setup consisted of a degasser, a binary pump, and a diode array detector. A Phenomenex Luna C18 column (4.6 mm × 250 mm, 5 µm) was used. Two solvents made up the mobile phase: Solvent B was a mixture of solvent A and acetonitrile (60:40, v/v) and Solvent A was a mixture of water and formic acid (99:1, v/v). The phenolic compounds were extracted using the following conditions: a flow rate of 0.5 mL/min at 25°C, isocratic conditions with 0% B from 0 to 5 minutes, and gradient conditions for the following steps: from 0% to 5% B in 20 minutes, from 5% to 15% B in 18 minutes, from 15% to 25% B in 14 minutes, from 25% to 50% B in 31 minutes, from 50% to 100% B in 3 minutes. After the extraction process, the column was washed and reconditioned. The temperature was kept at 25°C and the flow rate was set to 0.5 ml/min. UV-visible spectra were recorded for all peaks between 200 nm to 800 nm (Tanrıseven et al., 2020). By comparing each compounds UV spectra and retention durations to real standards and was identified. Additionally, an Agilent 6430 LCMS/MS spectrometer equipped with an electrospray ionization source was used to confirm the chemicals. The detection process for electrospray ionization mass spectrometry (ESI-MS) was optimized and executed in negative ion mode. Using authentic standards and the external standard approach, the chemicals were quantified and following Sonmezdag et al. (2019) approach, the phenolic content was calculated. To measure each phenolic compound, we used the calibration curves of the standard phenolic compounds. However, as it was not feasible to provide a standard substance for all compounds, we made use of calibration curves prepared with structurally comparable chemicals to quantify these compounds. The performance of the method developed was determined by using standard solutions, spiked and non-spiked samples (Barwick, 2016). The method was fully validated in terms of linearity, accuracy (recovery), inter-day and intra-day precision (repeatability), limits of detection and quantification (LOD/LOQ), and relative standard uncertainty (95% confidence level). The current chromatographic settings' detection (LOD) and quantification (LOQ) limits were calculated with signal-to-noise ratios (S/N) of about 3 and 10, respectively. Using commercial standards at concentrations often seen in microalgae samples (ranging from 1 to 100 mg/L) and with R^2 values over 0.995, standard curves were produced. As per the findings of Sonmezdag et al. (2019) and Tanrıseven et al. (2020), the measurements were conducted three times.

Determination of Sugar Profile by HPLC-RID

Extracts were injected into HPLC (Shimadzu Prominence-i LC-2030C) with a RID detector to detect and quantify sugar profiles. The analysis was conducted using a BIORAD Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad; Hercules, CA, USA) with a flow rate of 0.5 mL/min and H2SO4 (5 mM) as the carrier phase. To find the amounts of the compounds, solutions were made at five different concentrations for each reference substance, and the amounts of the compounds were computed using calibration curves (Lee & Coates, 2000).

Statistical Analysis

Statistical analysis was performed by One-Way ANOVA using SPSS 22.0 (version 22, SPSS Inc., Chicago, IL, USA). Duncan's test measured differences in the content levels of the results and means with *p*-values less than 0.05 were indicated to be statistically significant.

Results and Discussion

Antioxidant Activity and Total Phenolic Content

S. platensis is recognized as a rich source of nutritious phenolic and flavonoid compounds due to its higher production capacity than traditional plant-based sources. *S. platensis* contains phycocyanin and phenolic compounds which are known to have antioxidant properties. Two methods, DPPH and ABTS, were used to determine the antioxidant activity of the samples. The antioxidant capacity and total phenolic content of the samples are shown in Table 1. According to DPPH and ABTS methods, *S. platensis* extracts (2195.83 µmol Trolox /100g DW, and 2150.69 µmol Trolox /100g DW) applied to freeze-thawing technique was determined approximately 1.9 times higher antioxidant activity than ultrasonic-assisted conventional extraction technique (1143.75 µmol Trolox /100g DW, and 1099.30 µmol Trolox /100g DW) ($p<0.05$).

In Table 1 shows the total phenolic content (TPC) of samples extracted by freeze-thawing and ultrasonic-assisted conventional techniques. Significant differences existed in the total phenolic matter amounts between the samples ($p<0.05$). The freeze-thawing sample of *S. platensis* was showed the highest TPC, measuring 726 mg GAE/100g DW. When comparing the total phenolic matter results, it was found that the freeze-thawing process resulted in 1.9 times higher phenolic content than the sample subjected to ultrasonic-assisted classical extraction, which measured at 326.22 mg GAE/100 g DW. In the freeze-thawing technique, ice crystals are formed during the freezing process, causing the cells to swell and eventually break down, followed by shrinkage of the cells during thawing. Thus, it is thought that the release of bioactive compounds in the structure is facilitated and the amount of antioxidant capacity and phenolic compounds increases.

A study was conducted to analyze the impact of various drying methods on physical properties, the DPPH capacity of *S. platensis* was determined as 69.82 mg/100g DW (Kuatrakul et al., 2017). Uzlasir et al. (2023) determined the antioxidant capacity of *S. platensis* grown using different salt concentrations as 137-173 mM Trolox/g DW by DPPH radical scavenging capacity method and 373-655 mM Trolox/g DW by ABTS capacity method. It was determined that antioxidant capacity decreased with increasing salt concentration. In the study conducted by Martins et al. (2023), it was found that the TPC of extracts obtained from *S. platensis* using ultrasonic-assisted extraction was 36.50 mg GAE/g DW. Additionally, the antioxidant activity of the extracts was found to be 37.98 mg Trolox/ g DW.

Phenolic Compounds

Phenolic compounds are divided into different subgroups, such as phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids according to their chemical structures (De la Rosa et al., 2019).

Table 1. Antioxidant activity and total phenolic content of *S. platensis* extracted by different extraction techniques

Analyses	F&T	UACE	Sig.
DPPH (µmol Trolox /100g DW)	2195.83±69.90	1143.75±75.06	*
ABTS µmol Trolox /100g DW)	2150.69±9.50	1099.30±4.21	*
TPC (mg/100 g DW)	726.00±7.09	368.22±4.02	*

(*) The symbol in the row indicates statistical differences ($p<0.05$*); TPC: Total phenolic content; F&T: Freeze&Thawing; UACE: Ultrasonic-assisted conventional extraction

Table 2. Phenolic profile and amounts of *S. platensis* extracts by HPLC-ESI-MS/MS (mg/100g DW)

No	R_T	Compounds	$[M-H]^-$ /$[M-H]^{+*}$	MS^2	F&T	UACE	Sig.
1	4,1	Catechin (isomer1)	289[a]	267/245/172.9/154.9	0.15±0.03	0.39±0.05	*
2	4,89	Resveratrol C-hexoside	389[a]	293/210/147/96	1.41±0.03	0.15±0.00	*
3	5,93	Catechin (isomer 2)	289[a]	267/245/172.9/154.9	1.74±0.05	0.49±0.14	*
4	6,25	Mirisetin	317[a]	179/151/137/107	1.28±0.03	0.61±0.02	*
5	11,51	Ferulic acid	195[b]	177/145	5.84±0.73	3.76±0.49	*
6	12,32	Ferulic acid 4-O-glucuronide	369[a]	193/178	10.73±0.25	8.41±0.52	*
7	14,09	Gallic acid	169[a]	125	0.38±0.01	0.59±0.02	*
8	18,62	Phloroglucinol	127[b]	108	2.01±0.02	2.72±0.02	*
9	29,45	Brevifolin carboxylate	291[a]	219/174	9.13±0.03	5.12±0.22	*
10	44,16	Lutein	569[b]	551/533/578/495/119/145/121	0.38±0.00	0.21±0.01	*
				Total	34.06±3.79	22.45±2.78	*

(*) The symbol in the row indicates statistical differences ($p<0.05$*); R_T: Retention time; a: Negative ion mode, b: Positive ion mode; F&T: Freeze-Thawing; UACE: Ultrasonic-assisted conventional extraction

Table 3. Sugar profile of *S. platensis* extracts by HPLC-RID (mg/100g DW)

Peak no	R_T	Compounds	F&T	UACE	Sig.
1	9,30	Sucrose	93.71±4.09	46.81±3.68	*
2	10,88	Glucose	131.74±2.30	115.77±5.90	*
3	11,65	Fructose	8.45±0.10	4.36±0.50	*
		Total	233.89±1.70	166.94±6.49	*

(*) The symbol in the row indicates statistical differences ($p<0.05$*), R_T: Retention time; F&T: Freeze-Thawing; UACE: Ultrasonic-assisted conventional extraction

Phenolic compounds such as flavonoids and phenolic acids have antioxidant potential and their amounts vary according to the microalgae species and growing conditions. Table 2 shows the phenolic profile and amounts of *S. platensis* extracts. In addition to ferulic acid 4-O-glucuronide and brevifolin carboxylate, an isocoumarin derivative, as the dominant phenolic compound in *S. platensis* extracts, a total of 10 phenolic compounds including catechin isomer, resveratrol C-hexoside, myricetin (LOD-LOQ: 0.30-0.11 g/mL, R^2: 0.995), ferulic acid (LOD-LOQ: 0.18–0.60 g/mL, R^2: 0.995), gallic acid (LOD-LOQ: 1.89–6.30 g/mL, R^2: 0.995), phloroglucinol (LOD-LOQ: 0.32–0.44 g/mL, R^2:0.995) and lutein (LOD-LOQ: 0.08–0.28 g/mL, R^2: 0.995) were identified and quantified. Total phenolic compounds were 1.5 times higher in the freeze/thawing process than in the ultrasonic-assisted classic extraction technique ($p<0.05$). Catechins are important secondary metabolites found in plants and belong to the group of flavan-3-ols (or simply flavanols), which are part of the chemical family of flavonoids. Catechin is a molecule that has four diastereoisomers. Two of the isomers are in the *trans* configuration and are called catechin. The other two are in the *cis* configuration and are called epicatechin (Bernatoniene & Kopustinskiene, 2018; Tsuchiya, 2001). Catechin and epicatechin are known as the building blocks of proanthocyanidins, which are a type of condensed tannins. As flavonoids, catechins can act as antioxidants, but their antioxidant potential is relatively low when compared to other flavonoids, especially at lower concentrations. The ability to quench singlet oxygen is related to the catechin's chemical structure, a catechol moiety in the B ring, and a hydroxyl group in the C ring that activates the double bond (Pietta, 2000). With a colorless or slightly yellow crystalline form, gallic acid is one of the most prevalent phenolic acids in the kingdom of plants and finds widespread application in the food and pharmaceutical industries. It has been reported to have neuropsychological, metabolic, therapeutic activities, and cardiovascular disorders, including anti-inflammatory, antineoplastic, and antioxidant properties (Choubey et al., 2015). Lutein is a yellow-colored organic compound known as a vitamin carotenoid found in plants, which is covalently bonded with fatty acids. It is found in many organisms, including plants, yeasts, bacteria, and algae (Ochoa Becerra et al., 2020). Phloroglucinol is a naturally occurring secondary metabolite in certain plant species (Wong & Morita, 2019). The LC-ESI-MS/MS chromatograms of the phenolics identified in *S. platensis* samples are shown in Figure 1.

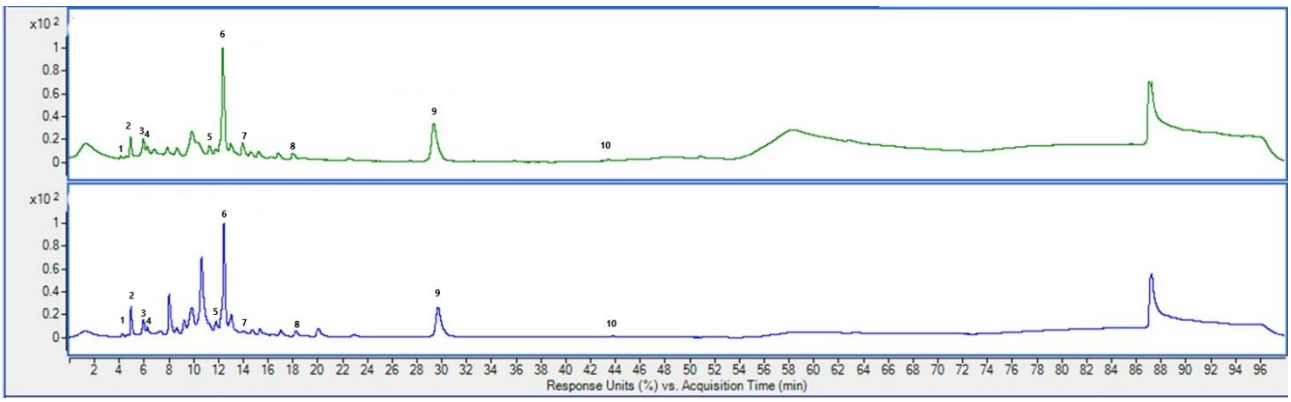

Figure 1. LC-ESI-MS/MS chromatograms of phenolic compounds of *S. platensis*
(1: Catechin (isomer1), 2: Resveratrol C-hexoside, 3: Catechin (isomer 2), 4: Mirisetin, 5: Ferulic acid, 6: Ferulic acid 4-O-glucuronide, 7: Gallic acid, 8: Fluoroglusinol, 9: Brevifolin carboxylate, 10: Lutein) (x: mAU, y: Aquisition time (min))

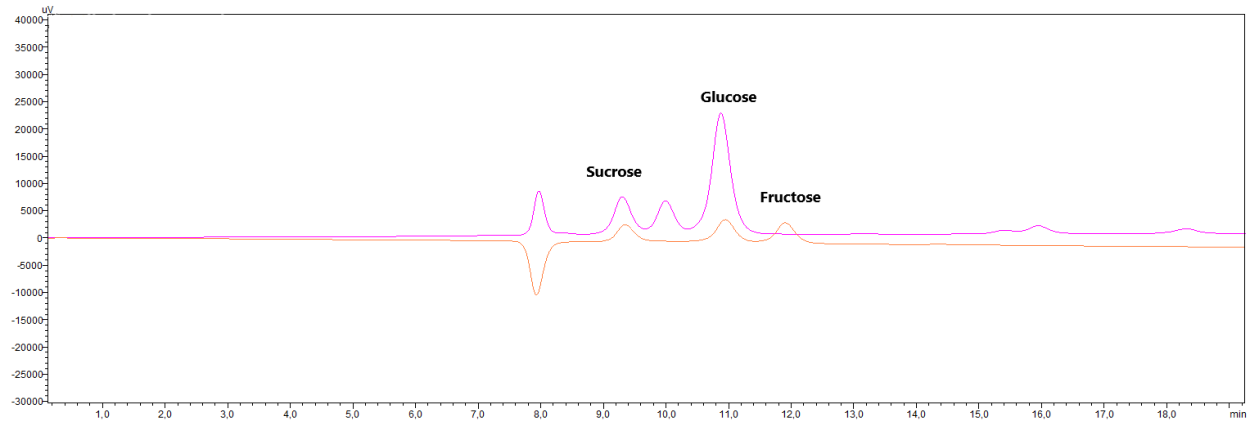

Figure 2. HPLC-RID chromatograms of the sugar profile of *S. platensis*

These natural phenolic compounds likely have a diverse range of chemical and biological properties, such as antioxidant and free radical-scavenging activities. Therefore, the total phenolic content was measured and correlated with the antioxidant capacity results of the samples. All the differences between the samples were found to be statistically significant. In our previous study in which we examined the effect of salt stress on the growth of *S. platensis*, a total of 24 phenolic compounds (catechin isomer, phloroglucinol, lutein, etc.) were identified, and their concentrations varied between 73 and 124 mg/100 g DW. A positive and strong correlation was found between antioxidant capacity and the amounts of total phenolic compounds (Uzlasir et al., 2023). Seghiri et al. (2019), discovered the presence of phenolic compounds like resveratrol, gallic acid, catechin, etc. in samples of *S. platensis*. They found that these compounds are usually found in extracts with higher polarity and are linked to antioxidant activity or a synergistic effect through their redox properties.

Sugar Profile

In general, microalgae contain approximately 10% carbohydrates on a dry weight basis. Microalgae species contain varying amounts and types of carbohydrates, with rhamnose, xylose, glucose, and mannose being the most abundant monomers (Villarruel-López et al., 2017). Sucrose, glucose, and fructose were determined in *S. platensis* using different extraction techniques. Statistically significant differences between the sugar contents ($p<0.05$) and the total sugar content were determined as 233.89 mg/100g DW in the freeze-thawing technique and 166.94 mg/100g DW in the ultrasonic-assisted conventional extraction technique (Table 3). The predominant sugar was glucose for both samples, and its amount was determined to be 115-131 mg/100g DW. The sugar chromatogram was given in Figure 2. Microalgae such as *Chlorella*, *Dunaliella*, *Nannochloropsis*, and *Spirulina* contain oligo and polysaccharides that make them potential prebiotics (Caporgno & Mathys, 2018; Gupta et al., 2017). In a study with commercial *Spirulina* by Al-Dhabi & Valan Arasu (2016), it was reported that the total sugar content of thirty-seven different *Spirulina* samples ranged between 309 and 1221 mg/100 g DW, and galactose, rhamnose, glucose, xylose, ribose, and fructose were detected as sugars. In another study, it was reported that rhamnose constitutes 53% of the total sugar in *Spirulina*, in addition to ribose, xylose, maltose, mannose, galactose, and glucose (Chaiklahan et al. 2013).

Conclusions

Spirulina platensis is a potential source of bioactive compounds, total phenolic content, and antioxidants with documented nutritional, physiological, and pharmacological benefits. Different extraction processes should be investigated to utilize these compounds more effectively. Green extraction processes are crucial to enhance efficiency, reduce processing time, and save energy. The antioxidant activity and total phenolic content

of the extracts prepared by the freeze-thawing technique were 1.9 times higher than those prepared by the ultrasonic-assisted conventional extraction technique. The most dominant sugar in both samples was glucose and the total sugar content was higher in the samples prepared by the freeze-thawing technique (233.89 mg/100 g DW) than by ultrasonic-assisted conventional extraction technique (166.94 mg/100 g DW). Ferulic acid 4-O-glucuronide and brevifolin carboxylate, an isocoumarin derivative, were determined as the dominant phenolic compounds in *S. platensis* extracts and the amount of total phenolic compounds was 1.5 times higher in the freeze/thaw method than in the ultrasonically assisted conventional extraction technique. *S. platensis* is known to have significant amounts of antioxidants, valuable secondary metabolites, and potential commercial applications and medicinal properties, but releasing these compounds is difficult due to the cell wall. This study determined that freeze-thawing can be a promising alternative to release bioactive compounds.

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Improvement of Seed Germination and Seedling Growth of Faba Bean (*Vicia Faba* L.) through Seed Priming

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ABSTRACT

In a lab experiment using seed priming, the faba bean (*Vicia faba* L.) seed germination and seedling development were studied. Twenty different priming techniques were utilized in the study, each comprising varying concentrations of NaOCI, $CaCl_2$, KNO_3, Manitol, PEG, KCL, H_2O and a control group that received no priming. Four replications of a completely randomized design (CRD) were used in the experiment. Among the three priming treatments, there were substantial differences in the seedling growth metrics and germination rate. When 500 ppm NaOCl was used as a treatment, the highest seed germination percentage (96%) was attained. Although 100 ppm PEG had the greatest germination index (42.92), 10000 ppm NaOCl had the quickest mean germination time (8.27). Additionally, at a concentration of 1500 ppm NaOCl, the greatest seedling vigor index (29.79) and maximum germination coefficient (12.28) were likewise obtained. With H_2O treatment, the maximum shoot length (21.09 cm) was observed for seedling growth parameters. The largest root length was produced by a 10000 ppm KNO_3 treatment (11.19 cm). With 20000 ppm KNO_3, the maximum root dry weight was achieved (88.50 mg), whereas H_2O produced the highest shoot dry weight (51.0 mg). Additionally, it was discovered that a treatment with 10000 ppm KNO_3 had the best root-shoot ratio (0.72). The research thus supports the possible use of seed priming as a method to improve faba bean seed germination and seedling growth. NaOCl and KNO_3 seemed to work best for faba bean seed germination and seedling growth.

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Introduction

Only a few species of leguminous crops are now exploited in agriculture, despite the fact that they have a significant role in dietary needs for both human and animals (Cernay et al., 2016; Mouradi et al., 2018). The faba bean (*Vicia faba* L.), which has a high nutritional value and is a great source of proteins, complex carbohydrates, dietary fiber, choline, lecithin, minerals, and secondary metabolites such as phenolics, distinguishes it as a unique legume crop (Paul and Gupta, 2021; Roy et al., 2022). Faba beans is an excellent crop for cropping systems because of its remarkable ability to symbiotically fix atmospheric N_2, which is highly dependent on sufficient populations of productive rhizobia that can extract N from both soil and the environment (Singh and bhatt, 2012).

One of the greatest and least expensive sources of lysine-rich protein is the green cotyledon of faba beans which is mostly consumed as a vegetable. It is the world's fourth-largest pulse crop after dry beans, dry peas and chickpeas making it one of the effective methods to combat hunger, especially in underdeveloped nations. Asia accounts for 41% of output on a global scale, followed by Africa and Europe and more than 66 nations throughout the world produce it (Paul and Gupta, 2021). It has a high concentration of L-dopa, which is a medication used to treat Parkinson's disease (Ramirez-Moreno et al., 2015). The faba bean also referred to as Kalimator, Baklakalai, and Bhograkalai locally, is cultivated in a few specific areas in Bangladesh's Central and Northern regions during the *rabi* season (Paul et al., 2022). This bean requires less input to flourish in less rich soil than other typical legume crops. Germination failure or in rare cases, a hard seed coat, is the major problem with faba bean farming in the field.

Seed priming is a method for partly hydrating non radicle emergence seeds according to Farooq et al. (2007). Priming seeds is largely used to increase uniformity and germination of different crops in unfavourable settings. Priming increases the viability of the seed and is typically used to get a straight, healthy crop stand (Draganic and Lekic, 2012). The water that was consumed by the primed treated seeds during priming was sufficient for hydration and was necessary for internal metabolic functions. Seeds that had been primed had a speedier germination time than seeds that hadn't been primed (Dezfuli et al., 2008). Compared to non-primed seed, priming treatments hasten emergence, increase the size of seedling shoots and increase fresh weight (Shim et al., 2009). Priming treatments had a considerable influence on germination and other growth metrics for seedling development as well as on the seedlings' ability to survive in the field (PK Roy et al., 2013; Dey et al., 2013).

Numerous methods of priming have been applied to synchronize and speed up seed germination. Hormone priming, hydro priming, osmo-conditioning, osmo-hardening, the use of ascorbate, solid matrix and wetting and drying primers are examples of typical techniques, according to Farooq et al. (2009). After seed priming, the seed's antioxidant activity increased. Seed priming enhances plant performance, according to several research findings, germination rates, synchronizes germination and produces seedlings that are strong and resistant to weed competition (Kaya et al., 2006; Basra et al., 2005). The goal of the study was to identify the most effective seed priming method with the goal of increasing faba bean seed germination and seedling vigor from this angle.

Materials and Methods

Description of the Experimental Site

At the Agro Innovation Laboratory, Department of Agronomy, Bangladesh Agricultural University, the laboratory test was conducted in January 2022. The testing location was located at latitude 23°22' N and longitude 90°33' E, 18 meters above sea level, and in a subtropical environment. The Laboratory received plant materials from the Bangladesh Agricultural Research Institute (BARI). Until they were utilized, seeds were kept in the fridge at 5°C in sealed containers. The seeds had an initial moisture content of about 10%, according to the oven drying process. Six priming agents of high laboratory quality were used in the experiment (Table 1).

Experimental Treatments and Design

One factor i.e. seed priming technique, was used in the experiment. Twenty techniques of seed priming were: i) 500 ppm NaOCl, ii) 1000 ppm NaOCl, iii) 1500 ppm NaOCl, iv) 10000 ppm $CaCl_2$, v) 20000 ppm $CaCl_2$, vi) 30000 ppm $CaCl_2$, vii) 10000 ppm KNO_3, viii) 15000 ppm KNO_3, ix) 20000 ppm KNO_3, x) 40000 ppm Manitol, xi) 60000 ppm Manitol, xii) 80000 ppm Manitol, xiii) 50 ppm PEG, xiv)100 ppm PEG, xv) 150 ppm PEG, xvi) 10000 ppm KCl, xvii) 20000 ppm KCl, xviii) 30000 ppm KCl, xix) H_2O and xx) Control (no priming). The experiment which was set up using a completely randomized design (CRD) included four replications.

Seed Priming

Individual faba bean seeds were submerged for 18 hours while using different priming agent solutions (previously produced with distilled water) at room temperature (25±2°C). The weight of the seeds to the volume of the solution was 1:5 (g/mL). The seeds were then removed from the priming agent solution and washed repeatedly in distilled water to remove any last bits of chemical residue. To get the seeds as near to their initial moisture content as possible, they were then forced-air dried for 36 hours at 28±2°C. Before being used for germination in the control treatment which did not receive any prior seed priming, the dried seeds were kept in sealed polythene bags and kept in a refrigerator at 5±1°C for 10 days.

Preparation of Germination Media and Seed Placement

Petridishes with measurements of 12 cm in diameter and 5 cm in depth were employed as the container, and sterilized sand was used as the germination media. The medium was irrigated with distilled water every morning to maintain a moisture level of around 80% of the field capacity. Fifty seeds were placed in 0.5 cm of moist sand in each petridish. Petridishes were placed on the laboratory desk at room temperature (25±2°C temperature, 70±5% relative humidity) with 11/13 h of light and darkness.

Procedure of Data Collection

Seed germination was monitored daily and final measurements were taken 14 days after sowing (DAS). Twenty seedlings were removed from each pot at 14 DAS in order to get data on seedling growth.

Germination Percentage (%)

The germination rate is the proportion of seeds that actually sprout from the quantity of seeds sown overall. The germination rate (GP) was determined using the following formula.

$$\text{GP: } \frac{\text{No. of germinated seeds at final count}}{\text{No. of seeds sown}} \times 100$$

Table 1. Information about the priming agents

SL. No.	Priming agent	Chemical formula	Manufacturer
1	Sodium hypochlorite	NaOCl	MERCK, India
2	Calcium chloride	$CaCl_2$	MERCK, India
3	Potassium nitrate	KNO_3	MERCK, India
4	Manitol	$C_{12}H_{24}O_{11}$	MERCK, India
5	Polyethylene glycol	PEG	LOBAL Chemie, India
6	Potassium chloride	KCl	MERCK, India

Mean Germination Time (MGT)

The following equation was used to get the mean germination time (Ellis and Roberts, 1981).

$$\text{Mean germination time} = \frac{\sum Dn}{\sum n}$$

Where, n is the number of seeds that germinated on day D and D is the number of days counting backward from the day germination began.

Germination Index (GI)

Time is estimated (in days) using the Germination Index (GI). It can only occur if a particular germination percentage is met. The Association of Official Seed Analysis used the following procedure to calculate the germination index (GI).

$$\text{GI} = \frac{\text{NGS}}{\text{DFC}} + \cdots + \frac{\text{NGS}}{\text{DLC}}$$

NGS: Number of germinated seeds
DFC: Days of first count
DLC: Days of final count

Seedling Vigor Index (SVI)

$$\text{SVI} = \frac{\text{Seedling length (cm)} \times \text{Germination percentage}}{100}$$

Where, seedling length = Root length + Shoot length

Germination Co-efficient (GC)

Using the formula shown below, the co-efficient of germination was computed (Copeland, 1976).

$$\text{Germination co-efficient} = \frac{N100(A1+A2.....An)}{A1T1+A2T2+\cdots.....AnTn}$$

Where, T is the time that corresponds to A, where n is the number of days left before the final tally and A is the number of seeds that have already sprouted.

Shoot Length

The length of the shoot, stated in centimetres, was calculated from the seedling's the biggest leaf measured from base to tip.

Root Length

The length of the roots indicated in centimetres was calculated from the seedling's the biggest leaf measured from base to tip.

Shoot Dry Weight

After drying the shoots of 20 seedlings used in the sample in a 60°C oven for 72 hours, the dry matter of the shoots was measured. Finally, each seedling's shoot dry weight was computed and represented in mg.

Root Dry Weight

After drying the roots of 20 seedlings for 72 hours at 60°C in an oven, root dry matter was calculated. Each seedling's root dry weight was ultimately estimated and reported in mg.

Root-Shoot Ratio

The ratio of root length to shoot length was used to calculate the root-shoot ratio.

Statistical Analysis

The recorded information was compiled and summarized for statistical analysis. Using the computer program MSTAT, an analysis of variance (ANOVA) was carried out. The mean differences between the treatments using the Duncan's Multiple Range Test (Gomez and Gomez (1984) with a 5% level of significance.

Results

Germination Percentage

The seed priming technique has a considerable impact on the faba bean's germination percentage (Figure 1). With the exception of KCl priming, all priming techniques led to increased germination rates. When seeds were primed with 500 ppm NaOCl, the final germination percentage of faba beans was found to be (96%), which was statistically comparable to those recorded with many other priming methods like 10000, 20000, and 30000 ppm $CaCl_2$, control, 1500 ppm KCl, any concentration of NaOCl, 10000 ppm KNO_3, and 150 ppm PEG. The lowest germination rate (64%) was obtained while priming with 30000 ppm KCl. No priming resulted in (94%) germination.

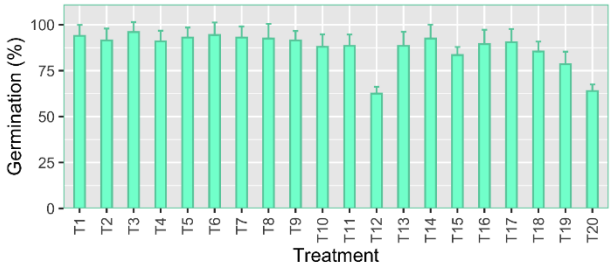

Figure 1. Germination percent of faba bean as influenced by seed priming method

T_1 = Control; T_2 = H_2O; T_3 = NaOCl (500 ppm); T_4 = NaOCl (1000 ppm); T_5 = NaOCl (1500 ppm); T_6 = $CaCl_2$ (10,000 ppm); T_7 = $CaCl_2$ (20,000 ppm); T_8 = $CaCl_2$ (30,000 ppm); T_9 = KNO_3 (10,000 ppm); T_{10} = KNO_3 (15,000 ppm); T_{11} = KNO_3 (20,000 ppm); T_{12} = Manitol (40,000 ppm); T_{13} = Manitol (60,000 ppm); T_{14} = Manitol (80,000 ppm); T_{15} = PEG (50 ppm); T_{16} = PEG (100ppm); T_{17} = PEG (150 ppm); T_{18} = KCl (10,000 ppm); T_{19} = KCl (20,000 ppm); T_{20} = KCl (30,000 ppm)

Mean Germination Time

The technique seed priming has a substantial impact on the germination period of faba beans (Table 2). The longest mean germination time was achieved with no priming (control) when compared to all other priming techniques. When seeds were not primed, the final mean germination period of faba beans was shown to be the longest (9.22 days). The shortest mean germination time (8.27 days) was obtained after priming with 1000 ppm NaOCl, followed by 1500 ppm NaOCl, H_2O, 100 ppm and 150 ppm PEG, and 10000 ppm and 20000 ppm $CaCl_2$.

Germination Index

The seed priming procedure had a considerable impact on the faba bean's germination index (Figure 2). The ultimate germination index of faba beans was found to be the greatest (96.93) when seeds were primed with 1500 ppm NaOCl. This value was statistically comparable to those reported with numerous other priming techniques, including H_2O, 20000 ppm $CaCl_2$, 500 ppm, and 1000 ppm NaOCl. The least favorable germination index (45.51) was obtained after priming with 30000 ppm KCl.

Seedling Vigor Index

The method of seed priming had a considerable impact on the seedling vigor index of the faba bean (Table 2). When seeds were primed with 1500 ppm NaOCl, the faba bean's ultimate seedling vigor index was found to be at its greatest value (29.79), which was statistically comparable to results obtained with several other priming techniques, such as 500 ppm NaOCl, H_2O, and $CaCl_2$ at any concentration. The lowest seedling vigor index (13.15), which was statistically comparable, was obtained after priming with 30000 ppm KCl, followed by priming with 40000 ppm Manitol and 20000 ppm KCl.

Germination Co-efficient

The seed priming technique has a considerable impact on the faba bean's germination coefficients (Table 2). The ultimate germination coefficient of faba bean was found to be the greatest (12.28) when seeds were primed with 1500 ppm NaOCl, which was statistically comparable to those reported with several other priming techniques such 150 ppm PEG, 1000 ppm NaOCl, and H_2O. The lowest germination coefficient (11.01) from priming with no priming technique control was statistically comparable to that of priming with 15000 ppm KNO_3 and 30000 ppm KCl.

Shoot Length

The seed priming strategy has a substantial impact on faba bean shoot length (Table 3). When seeds were primed with water, the ultimate shoot length of the faba bean was found to be the highest (21.09 cm), which was statistically comparable to those recorded with several other priming techniques including 500 ppm and 1000 ppm NaOCl, 20000 ppm $CaCl_2$, and 50, 500, and 150 ppm PEG, respectively. The lowest shoot length (11.14 cm) was statistically obtained while priming with 30000 ppm KCl, which was statistically followed by priming with 40000 & 80000 ppm Manitol, 20000 ppm KCl, 1500 ppm NaOCl, and 10000 ppm $CaCl_2$.

Root Length

The seed priming technique had a substantial impact on the length of the faba bean roots (Table 3). The maximum roots length (11.19 cm) of the faba bean was discovered when seeds were primed with 10000 ppm KNO_3 which was statistically comparable 20000 ppm KNO_3. The lowest roots length of faba bean (7.08 cm) after priming was achieved with 30000 ppm KCl.

Shoot Dry Weight

The seed priming strategy had a considerable impact on the shoot dry matter of the faba bean (Figure 3). When seeds were primed with water, the ultimate shoot dry weight of the faba bean was discovered to be the greatest (51.00 mg), which was identical to those reported with several other priming techniques like 10000 ppm NaOCl and 20000 ppm $CaCl_2$ correspondingly. The lowest shoot dry matter (26.50 mg) was produced by priming with 30000 ppm KCl, which was statistically equal to priming with 80000 ppm Manitol.

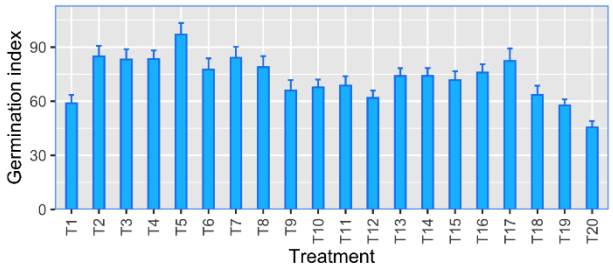

Figure 2. Germination index of faba bean as influenced by seed priming

T_1 = Control; T_2 = H_2O; T_3 = NaOCl (500 ppm); T_4 = NaOCl (1000 ppm); T_5 = NaOCl (1500 ppm); T_6 = $CaCl_2$ (10,000 ppm); T_7 = $CaCl_2$ (20,000 ppm); T_8 = $CaCl_2$ (30,000 ppm); T_9 = KNO_3 (10,000 ppm); T_{10} = KNO_3 (15,000 ppm); T_{11} = KNO_3 (20,000 ppm); T_{12} = Manitol (40,000 ppm); T_{13} = Manitol (60,000 ppm); T_{14} = Manitol (80,000 ppm); T_{15} = PEG (50 ppm); T_{16} = PEG (100ppm); T_{17} = PEG (150 ppm); T_{18} = KCl (10,000 ppm); T_{19} = KCl (20,000 ppm); T_{20} = KCl (30,000 ppm)

Table 2. Seed germination, seedling vigor and germination co-efficient of faba bean as influenced by seed priming method.

Treatment	Mean germination time (days)	Seedling vigor index	Germination co-efficient
T_1	9.22a	27.75a-d	11.01d
T_2	8.46f-h	28.95ab	11.80ab
T_3	8.64c-h	29.76a	11.58bc
T_4	8.27h	27.57a-d	11.80ab
T_5	8.33gh	29.79a	12.28a
T_6	8.65c-h	28.58ab	11.52b-d
T_7	8.58d-h	28.20a-c	11.75b
T_8	8.73b-g	25.09b-e	11.45b-d
T_9	9.00a-c	22.61e-g	11.13cd
T_{10}	9.02a-c	23.80d-f	11.06cd
T_{11}	8.82a-f	27.04a-d	11.32b-d
T_{12}	8.77b-f	15.38h	11.35b-d
T_{13}	8.90a-e	24.11c-f	11.31b-d
T_{14}	8.74b-g	20.85fg	11.45b-d
T_{15}	9.13ab	24.16c-f	11.16cd
T_{16}	8.64c-h	25.41b-e	11.33b-d
T_{17}	8.51e-h	26.24a-e	11.81ab
T_{18}	9.02a-c	23.84d-f	11.18cd
T_{19}	8.73b-g	19.63g	11.50b-d
T_{20}	8.94a-d	13.15h	11.07cd
$S\bar{x}$	0.06	1.01	0.07
Sig. level	**	**	**
CV (%)	2.85	10.58	2.86

Different letters in the same column indicated significant differences at 5 % level of probability; ** 1% level of significance. T_1 = Control; T_2 = H_2O; T_3 = NaOCl (500 ppm); T_4 = NaOCl (1000 ppm); T_5 = NaOCl (1500 ppm); T_6 = $CaCl_2$ (10,000 ppm); T_7 = $CaCl_2$ (20,000 ppm); T_8 = $CaCl_2$ (30,000 ppm); T_9 = KNO_3 (10,000 ppm); T_{10} = KNO_3 (15,000 ppm); T_{11} = KNO_3 (20,000 ppm); T_{12} = Manitol (40,000 ppm); T_{13} = Manitol (60,000 ppm); T_{14} = Manitol (80,000 ppm); T_{15} = PEG (50 ppm); T_{16} = PEG (100ppm); T_{17} = PEG (150 ppm); T_{18} = KCl (10,000 ppm); T_{19} = KCl (20,000 ppm); T_{20} = KCl (30,000 ppm)

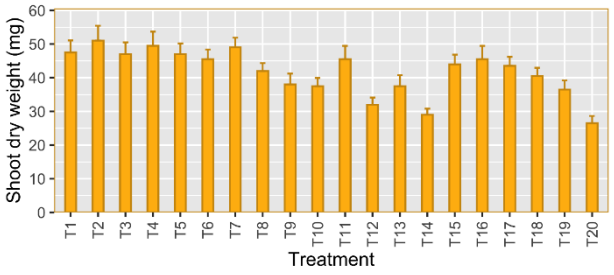

Figure 3. Shoot dry weight of faba bean as influenced by seed priming

T_1 = Control; T_2 = H_2O; T_3 = NaOCl (500 ppm); T_4 = NaOCl (1000 ppm); T_5 = NaOCl (1500 ppm); T_6 = $CaCl_2$ (10,000 ppm); T_7 = $CaCl_2$ (20,000 ppm); T_8 = $CaCl_2$ (30,000 ppm); T_9 = KNO_3 (10,000 ppm); T_{10} = KNO_3 (15,000 ppm); T_{11} = KNO_3 (20,000 ppm); T_{12} = Manitol (40,000 ppm); T_{13} = Manitol (60,000 ppm); T_{14} = Manitol (80,000 ppm); T_{15} = PEG (50 ppm); T_{16} = PEG (100ppm); T_{17} = PEG (150 ppm); T_{18} = KCl (10,000 ppm); T_{19} = KCl (20,000 ppm); T_{20} = KCl (30,000 ppm)

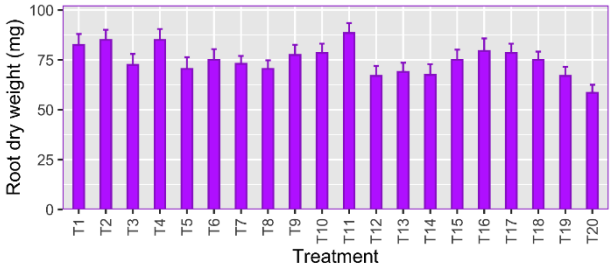

Figure 4. Root dry weight of faba bean as influenced by seed priming method

T_1 = Control; T_2 = H_2O; T_3 = NaOCl (500 ppm); T_4 = NaOCl (1000 ppm); T_5 = NaOCl (1500 ppm); T_6 = $CaCl_2$ (10,000 ppm); T_7 = $CaCl_2$ (20,000 ppm); T_8 = $CaCl_2$ (30,000 ppm); T_9 = KNO_3 (10,000 ppm); T_{10} = KNO_3 (15,000 ppm); T_{11} = KNO_3 (20,000 ppm); T_{12} = Manitol (40,000 ppm); T_{13} = Manitol (60,000 ppm); T_{14} = Manitol (80,000 ppm); T_{15} = PEG (50 ppm); T_{16} = PEG (100ppm); T_{17} = PEG (150 ppm); T_{18} = KCl (10,000 ppm); T_{19} = KCl (20,000 ppm); T_{20} = KCl (30,000 ppm)

Root Dry Weight

The seed priming approach had a considerable impact on the root dry matter of the faba bean (Figure 4). When seeds were primed with 20000 ppm KNO_3, the ultimate root dry weight of the faba bean was found to be the greatest (88.50 mg), which was statistically comparable to those recorded with several other priming techniques such H2O, 1000 ppm NaOCl, control, 10000 & 15000 ppm KNO_3, 100 & 150 ppm, respectively. The lowest root dry matter (58.50 mg) was obtained after priming with 30000 ppm KCl. This was followed by priming with 40000 & 80000 ppm Manitol and 20000 ppm KCl.

Root: Shoot

The seed priming technique has an impact on the root-shoot ratio of faba beans. According to (Table 3), the faba bean's root-shoot ratio was highest (0.72) when seeds were primed with 10000 ppm KNO_3 and lowest (0.43) when seeds were primed with 100 ppm PEG.

Discussion

The germination percentage was greater with all priming techniques except KCl (at any concentration). The highest final germination percentage of faba beans was observed when seeds were primed with 500 ppm NaOCl, which was statistically comparable to those recorded with other priming methods like 10000, 20000, and 30000 ppm $CaCl_2$, control, 1500 ppm KCl, any concentration of NaOCl, H_2O, 10000 ppm KNO_3, and 150 ppm PEG. The lowest germination rate was obtained after priming with 30000 ppm KCl. The frequency of germination with no priming was 94% (Table 2). Production of proteins and growth inhibitors being released (Khan et al., 1977), as well as the repair of deteriorating DNA in seeds (Di Girolamo and Barbanti, 2012), may be the causes of better germination by priming. According to Pukacka and Ratajczak (2005), priming stimulates antioxidant enzymes that minimize peroxidation in the seed and might hasten germination, maintaining seed vigor. According to Abbas et al. (2018), pressing dirt into a solid matrix for 12 hours and osmo priming with PEG-6000 are both recommended for enhancing crop germination and growth. Raun et al. (2002) found that KCl priming of rice seeds improved germination rates. According to Basra et al. (2005) and Ghiyasi et al. (2008), vitamin C priming can hasten and uniformize germination, which enhances germination and establishment in a number of field crops, including faba bean.

The sole method of treatment without priming or a control had the longest mean germination time of all the treatments. The results indicated that when the seeds were not primed using any other priming strategies, the faba bean's ultimate mean germination time was the longest. According to Farooq et al. (2010), the seed undergoes biochemical changes as a result of seed priming, including hydrolysis, enzyme activation and dormancy breaking which might significantly shorten the time it takes for a seed to emerge and boost its final germination rate. Karmore and Tomar (2015) found that seed priming over 24 hours resulted in a much quicker time for 50% emergence than untreated seeds, which needed the mean minimum germination time. The faba bean germination index changed equally regardless of the seed priming technique. The highest final germination index for faba beans was found when seeds were pre-treated with 1500 ppm NaOCl, which was statistically equivalent to those reported with several other priming strategies such H_2O, 20000 ppm $CaCl_2$, 500 ppm, and 1000 ppm NaOCl. While priming with 30000 ppm of KCl (Table 2), the lowest germination index was attained. It has been demonstrated that hydro-priming increases germination rate in a variety of crop species, especially under challenging growing environments (Jisha et al., 2018).

The final seedling vigor index for the faba bean was discovered to be at its highest when seeds were primed with 1500 ppm NaOCl. This result was statistically similar to those found with a number of different priming methods, including 500 ppm NaOCl, H_2O, and $CaCl_2$ at any concentration. As shown in (Table 2), priming with 30000 ppm KCl resulted in the lowest seedling vigor index, which was statistically followed by priming with 40000 ppm Manitol and 20000 ppm KCl. Seeds that were primed produced seedlings with a higher seedling vigor index than seeds that were not primed. Mahender et al. (2015) defined that the growth of robust seedlings in any form of environmental condition is known as seedling early vigor. When seeds were pre-treated with 1500 ppm NaOCl, the ultimate germination coefficient of faba beans was discovered to be at its highest level.

Table 3. Seedling growth of faba bean as influenced by seed priming method

Treatments	Shoot length (cm)	Root length (cm)	Root-shoot ratio
T_1	19.16a-d	10.36a-c	0.54c-e
T_2	21.09a	10.53ab	0.50de
T_3	20.47ab	10.53ab	0.52c-e
T_4	20.63ab	9.65a-c	0.47de
T_5	19.61a-c	9.93a-c	0.51c-e
T_6	19.39a-c	10.88ab	0.57cd
T_7	20.40ab	9.94a-c	0.49de
T_8	17.35b-e	9.78a-c	0.57cd
T_9	15.96d-f	11.19a	0.72a
T_{10}	16.51c-e	10.56ab	0.64a-c
T_{11}	19.44a-c	11.13a	0.58cd
T_{12}	15.60ef	9.03bc	0.59b-d
T_{13}	16.11d-f	9.01bc	0.57cd
T_{14}	13.25fg	9.31a-c	0.70ab
T_{15}	19.62a-c	9.38a-c	0.49de
T_{16}	19.91ab	8.51cd	0.43e
T_{17}	19.61a-c	9.27a-c	0.48de
T_{18}	17.83a-e	9.99a-c	0.56cd
T_{19}	15.92d-f	9.10bc	0.58cd
T_{20}	11.14g	7.08d	0.64a-c
$S\bar{x}$	0.60	0.22	0.02
Sig. Level	**	**	**
CV (%)	11.28	11.73	13.83

Different letters in the same column indicated significant differences at 5 % level of probability; ** 1% level of significance. T_1 = Control; T_2 = H_2O; T_3 = NaOCl (500 ppm); T_4 = NaOCl (1000 ppm); T_5 = NaOCl (1500 ppm); T_6 = $CaCl_2$ (10,000 ppm); T_7 = $CaCl_2$ (20,000 ppm); T_8 = $CaCl_2$ (30,000 ppm); T_9 = KNO_3 (10,000 ppm); T_{10} = KNO_3 (15,000 ppm); T_{11} = KNO_3 (20,000 ppm); T_{12} = Manitol (40,000 ppm); T_{13} = Manitol (60,000 ppm); T_{14} = Manitol (80,000 ppm); T_{15} = PEG (50 ppm); T_{16} = PEG (100ppm); T_{17} = PEG (150 ppm); T_{18} = KCl (10,000 ppm); T_{19} = KCl (20,000 ppm); T_{20} = KCl (30,000 ppm)

This number was statistically equivalent to the results obtained with a variety of alternative priming methods, such as 150 ppm PEG, 1000 ppm NaOCl, and H_2O. The priming with no priming technique control had the statistically lowest germination coefficient, which was followed by priming with 15000 ppm KNO_3 and 30000 ppm KCl (Table 2). According to Karmore and Tomar (2015), who discovered that seed priming procedures had a favorable impact on root and shoot length in comparison to untreated seed. Farooq et al. (2012) provided additional evidence for the current similar, increased ability of osmopriming with $CaCl_2$ to hasten germination, vigor index, and seedling vigor in spring maize.

The largest faba bean ultimate shoot length was discovered when seeds were water-primed. This outcome was statistically equivalent to numerous other priming methods, including 50, 500, and 150 ppm PEG, 20000 ppm $CaCl_2$, 500, and 1000 ppm NaOCl, and 500, 1000, and 20000 ppm $CaCl_2$. The statistical analysis revealed that priming with 30000 ppm KCl resulted in the lowest shoot length, which was then followed by priming with 40000 ppm and 80000 ppm Manitol, 20000 ppm KCl, 1500 ppm NaOCl, and 10000 ppm $CaCl_2$ (Table 3). According to research by Lee et al. (2000), seed priming affects plumule and radicle length more significantly under optimal soil moisture conditions than it does under insufficient or excessive soil moisture. The faba bean's ultimate shoot dry matter was discovered to be the highest when seeds were moistened. This result was statistically equivalent to other priming methods, such as 10000 ppm NaOCl and 20000 ppm $CaCl_2$, respectively. The priming with 30000 ppm KCl resulted in the statistically lowest shoot dry matter, which was followed by 80000 ppm Manitol (Table 3). The faba bean's final root dry matter was discovered to be the highest when seeds were pre-treated with 20000 ppm KNO_3. This result was statistically equivalent to those obtained with a number of different priming methods, including H_2O, 1000 ppm NaOCl, control, 10000 & 15000 ppm KNO_3, 100 & 150 ppm, etc. Priming with 30000 ppm KCl resulted in the statistically lowest root dry matter, which was statistically followed by priming with 40000 ppm & 80000 ppm Manitol and 20000 ppm KCl (Table 3). Different pea types and seed priming methods have an impact on the total dry weight (g/plant), shoot and root dry weights, and total dry weight (g/plant). It was observed that seed priming with H_2O_2 enhanced shoot dry weight after ten additional priming procedures, including the concentration of PEG, KH_2PO_4, H_2O_2, KNO_3, ABA, and hydropriming (Yanglem et al., 2021). The ratio of root and shoot of the faba bean was shown to be largest when seeds were pre-treated with 10000 ppm KNO_3 or 100 ppm PEG, and vice versa (Table 3).

Conclusion

Faba bean seed germination and seedling growth may be aided by seed priming according to studies which supports this theory. The optimal priming agent was found to be seed priming with NaOCl or KNO_3. These results point to new paths for the development of faba bean seed priming for improved seed germination and increased seedling growth under various stress situations to ensure good plant establishment, better growth and higher yield.

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Conflict of Interests

The authors certify that they have no financial or other competing interests to disclose with relation to the current work.

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Comparative Analysis of Soil Phosphorus Determination Methods and Their Correlation with Plant Phosphorus in Standing Wheat Crops

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ABSTRACT

This study compared the accuracy of various soil phosphorus assessment methods to measure the soil's ability to supply plants with phosphorus over a brief period in the field. Twenty individual soil samples were collected from a standing wheat *(Triticum aestivum L)* crop at depths ranging from zero to twenty centimeters. An equivalent plant spike sample was also procured from the soil sampling fields. In comparison to the wet acid digestion method used to detect phosphorus in plants, several methods were utilized to assess phosphorus in the soil, including resin extractable phosphorus, AB-DTPA extractable phosphorus, NaHCO3 extractable phosphorus, water-soluble phosphorus in suspension, and paste. The levels of variation and deficiency of phosphorus, which were found by different methods followed different patterns as shown by the fact that, AB-DTPA method finds phosphorus deficiency in 20% of samples while on the other hand, Olsen method finds phosphorus deficiency in 80% of samples. Even with such a small sampling area, none of the procedures showed a significant correlation with any other method that might account for uneven variation among the samples when determined by distinct procedures. However, corrections were observed to a certain degree between ammonium bicarbonate-diethylenetriaminepentaacetic acid (AB-DTPA) extractable and resin, as well as between other procedures and the plant P scale. Both resin and ammonium bicarbonate-diethylenetriaminepentaacetic acid (AB-DTPA) had a strong relationship with plant phosphorus, with the former showing a significant correlation of 0.48 and 0.21, respectively. Hence Resin and AB-DTPA methods are recommended for the determination of phosphorus under certain soil and plant conditions.

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Introduction

Wheat *(Triticum aestivum L.)* is prominent among the grains of the Poaceae family and is cultivated globally on a large scale. In Pakistan specifically, wheat is widely grown in various regions, especially in regions of Punjab. Wheat has always played a substantial role in the development of human civilization and is recognized as the most important staple grain all over the world. The grains of wheat are consumed directly or indirectly through human diets, while wheat straws serve as valuable fodder for animals. Most people over the globe consume wheat as a staple food and it provides 73% of the protein and calories they need. (Bashir et al., 2015).

Phosphorus serves an essential role as a micronutrient in sustaining life cycles. (Marschner, 2012; Ding et al., 2020; Hopkins, 2020). The availability of Phosphorus to the plant is reduced if the soil has high calcium content. A significant portion of phosphorus fertilizer when applied to the soil tends to bind to the calcite surfaces. This makes the Phosphorus temporarily available to the plants. This can potentially lead to a decrease in crop yield (Saeed et al., 2021). To determine the availability of plant's phosphorus (P) in the soil, several soil chemical parameters such as pH and limestone concentration play a significant role (Fixen and Bruulsema, 2014; Lindsay, 2001). Calcareous soils frequently lack a sufficient supply of plant-available phosphorus (P). This occurs because of the reduced solubility, which is caused by fixation and sorption, (Lindsay, 2001; Hopkins et al., 2014; (Boukhalfa-Deraoui et al., 2015; Hopkins, 2020; Mihoub and Boukhalfa-Deraoui, 2014). Hence this low solubility of Phosphorus reduces the efficiency of P fertilizer (Jamal et al., 2018; Mihoub and Boukhalfa-Deraoui, 2014). Thus, most

calcareous soils require significant exogenous P replenishment to maintain stable yields. Soil testing serves the purpose of guiding effective and efficient soil nutrient management by utilizing the correlations between soil test results and crop responses to applied nutrients. It is essential to observe that not all available extractants have a standard calibration between the number of extractables.

Testing the soil is a typical practice for determining nutrient needs and assessing current nutrient levels. This enables more precise nutrient prescription and enhanced nutrient utilization efficiency. However, most of these techniques are narrow in scope, requiring additional steps and resources to determine the concentration of a single nutrient. As a result, they are impractical for use in large-scale soil testing laboratories. Because of their capacity to extract many nutrients at once, they need the rapid assessment of many samples. Multi-nutrient extractants provide a realistic choice in the face of these challenges. (SHRIVASTAV et al., 2023). Commonly the practice of prescribing nutrients is prevalent and is employed. Mostly this practice is based on soil analysis. To achieve the intended yields, it is necessary to determine the appropriate nutrients. For this, it is necessary to assess the nutrient availability of the soil. The accuracy of any technique used to estimate nutrient availability in soils is affected by soil characteristics such as organic matter concentration, temperature, and mineral composition. Soil test values for individual nutrients and crop yield may not be reliably predicted by simple correlation coefficients alone when there are variations in both the soil and the applied nutrients under field conditions. Therefore, multiple regression analyses, considering all the essential nutrients (N, P, and K), can be used as an alternate tool for assessing the efficacy of various soil test methods simultaneously by using R2 values. The efficacy of several soil testing methods in practice has been assessed. Soil characteristics such as organic matter concentration, temperature, and mineral composition affects The accuracy of any technique, which is used to estimate nutrient availability in soils. (Velayuthan et al., 1984). The screening process plays a vital role in identifying the appropriate soil testing method (Mosi and Lakshminarayanan, 1985). Water-extractable P (WEP), calcium chloride-extractable P (CCEP), and Olsen-extractable P (OEP) can be used to extract the accessible P forms in soils, whereas total P can be seen as an indicator of the soil's Preserve and the accumulation of P owing to extensive P application. WEP and CCEP are used to assess P leaching from soils, while OEP is employed as an agronomic indicator. Since standard P extraction methods are thought to depict labile P fractions more accurately, it is important to do research into the P background level using these methods. In this study, we analyzed the relationship between plant phosphorus (P) levels and the characteristics of phosphorus (P) in the soil solution. The main goals of this study were focused on the wheat crop grown at the Research Farm in the Peshawar region of the University of Agriculture. Specifically, we aimed to investigate the connection between different forms of soil extractable phosphorus (AB- DTPA extractable P, Olsen P, water-soluble P, Resin extractable P, and paste extractable P) and the concentration of phosphorus in wheat plants.

Materials and methods

Soil and Plant Sample Collection

A study was conducted to maximize the determination and absorption of phosphorous (P) by plants to improve the yield and health of wheat. The experiment involved collecting samples from the Research Farm of the Agriculture University Peshawar during the spring season. Plant samples were obtained at the flowering i.e. the anthesis stage in wheat. The samples were subsequently dried for laboratory analysis. At each sampling site, 10 to 15 leaves were collected from the plants. Soil samples were also collected from a depth of 0-20 centimeters from three to four different sites at a single experimental location. These soil samples were mixed to create a composite soil sample, which was then combined with the plant samples from the respective site. Along with this, subsamples were taken at a variety of depths and then combined using stainless steel augers at each sampling site. These soil samples were then collected, sealed in labeled plastic bags and transported to a lab for testing.(Beygi and Jalali, 2018). Beygi and Jalali, (2018) conducted a study where they reported on the sampling and analysis of prevalent soil properties. Furthermore, GPS readings were recorded at each site for accurate location information. In the later processes a comparison was made between the plant and soil samples to assess the phosphorous concentration.

This comparison sought to ascertain the concentration of Soil-Solution-P in the leaves of wheat plants. The ultimate goal was to improve understanding and use of P-determination and absorption by plants, which would result in higher agricultural output and healthier plant characteristics.

Data Analysis

Several characteristics, including soil phosphorus (P) levels, were evaluated through a thorough examination of soil and plant samples in this study. Several methodologies were employed to ascertain the P levels, encompassing the AB-DTPA extractable P method proposed by (Havlin and Soltanpour, 1981), the Olsen P method as per Olsen (1954), water-soluble P determined using the paste extract technique outlined by Gardner, (1986), and resin-P based on Murphy and Riley's approach (1962). Additionally, the concentration of phosphorus in plant leaves was measured to complement the soil analysis. Water-soluble phosphorus content was measured by taking 1 gram of soil and vigorously mixing it with 5 millilitres of purified water for a duration of 30 minutes (Jalali et al., 2023). To calculate the Olsen Extractable Phosphorus (OEP), a soil sample weighing 1 gram was vigorously mixed with 20 milliliters of Olsen solution (0.5 M $NaHCO_3$) for a duration of 30 minutes (Olsen et al., 1982). The samples acquired using the aforementioned techniques underwent centrifugation and filtration. To analyse the total phosphorus content, 0.2 g of soil was subjected to furnace treatment at 550 ℃ for 1 hour. Following this, 25 ml of 1 N HCl was introduced, and the mixture was heated for 15 minutes (Andersen, 1976). Using a UV-visible spectrophotometer and the colorimetric method developed by (Murphy and Riley, 1962), the P concentration in each of the aforementioned extracts was determined.

Results

AB-DTPA extractable phosphorus

The soil's AB-DTPA extractable phosphorus (P) content ranges from 2.1 to 5.7 mg kg^{-1}, with an average value of 3.9 mg kg^{-1}. (Table.1) The standard deviation of the P content is low at 1.1, indicating a small variation among the samples. Among the samples analysed, 20% of the soils are deficient in P, while the remaining 80% have P levels in the low to medium range. When compared to the standard values in (Table. 2), there are insufficient P levels in any of the samples. The formation of complexes is responsible for P deficiency in calcareous soils.

$$\text{AB} - \text{DTPA P}\ (\text{mg kg}^{-1}) = \frac{\text{Readings} \times \text{voulme made}}{\text{Weight of soil}}$$

Olsen Extractable Phosphorus

Soil Olsen P levels varied from 4.2 to 11.2 mg kg^{-1} (mean: 7.7 mg kg^{-1}) with a small range and standard deviation of 1.8. (Table. 1) Out of the 20 samples, 80% were deficient in P, while the remaining 20% had medium levels. No soil samples indicated toxicity or adequate P levels.

Water Soluble Phosphorus in Paste and Suspension

The soil's water-soluble phosphorus (P) content ranged from 0.7 to 1.3 mg kg^{-1}, with an average value of 1.1 mg kg^{-1}. (Table. 1)The variation in water-soluble P values was relatively small, with a standard deviation of 1.1. Additionally, the paste P content in the soil ranged from 0.1 to 0.3 mg L^{-1}, with a mean value of 0.2 mg L^{-1}. The paste P values also showed a narrow range of variation, with a standard deviation of 0.04

Resin Extractable Phosphorus (Bioavailable-Phosphorus)

The soil's Resin P levels varied from 3.9 to 9.0 mg L^{-1} mg L^{-1}, averaging 5.0 mg L^{-1}. The range of Resin P values was relatively small, with a standard deviation of 1.5 (Table. 1).

Plant extractable Phosphorus

Plant P varied from 0.1% to 0.3%, averaging at 0.2% with a narrow range and a standard deviation of 0.05 (Table. 1).

Discussions

Extraction of P through AB-DTPA

The AB-DTPA extractable phosphorus (P) analysis of the soil shows a relatively narrow P content range, averaging 3.9 mg kg^{-1} with a low standard deviation of 1.1. This suggests the consistent availability of P. However, 20% of the soils are deficient in P, which negatively impacts plant growth and soil fertility. None of the samples meet the sufficient P levels indicated in Table 2, highlighting the need to address this deficiency for sustainable agriculture and higher crop yield. The deficiency in calcareous soils is caused by complex formation due to high pH levels, resulting in P immobilization. Implementing soil management strategies such as adjusting pH, adding organic matter, and using P fertilizers can improve P availability and nutrient uptake. Similarly, increasing the levels of applied P enhances the water-soluble AB-DTPA extractable P. AB-DTPA extractable phosphorus (P) concentration levels rise during initial growth stages, but in subsequent periods, P values can increase due to fixation and other complexation processes occurring gradually (Griffith, 1983).

Table 1. Concentrations of soil and plant phosphorus as determined by different protocols

S. No.	AB-DTPA P	Olsen P	Paste P	WS P	Resin P	Plant P
	mg kg-1		mg L-1	mg kg-1	mg kg-1	%
1	4.76	9.54	0.32	1.20	7.89	0.27
2	3.96	7.8	0.21	1.02	6.46	0.21
3	3.84	5.14	0.18	1.11	4.45	0.16
4	4.05	6.78	0.13	1.20	3.97	0.18
5	4.88	7.57	0.23	1.27	4..16	0.24
6	5.42	8.34	0.19	1.10	5.87	0.29
7	3.17	6.73	0.24	0.93	4.95	0.19
8	5.13	9.34	0.14	1.35	5.88	0.31
9	4.67	8.76	0.23	1.12	8.66	0.35
10	2.14	5.63	0.19	0.72	4.69	0.26
11	3.84	8.54	0.24	1.21	5.88	0.25
12	3.46	8.26	0.15	1.23	5.88	0.28
13	3.66	4.21	0.26	1.03	5.48	0.32
14	2.48	5.76	0.15	0.99	5.76	0.25
15	2.45	5.13	0.21	1.32	4.95	0.26
16	2.37	9.91	0.22	0.92	4.11	0.21
17	5.58	11.23	0.21	1.26	8.40	0.32
18	3.83	8.63	0.18	1.13	6.64	0.22
19	5.68	8.9	0.22	1.23	9.02	0.34
20	3	8.34	0.19	1.12	5.400	0.26
Min	2.1	4.2	0.1	0.7	3.9	0.1
Max	5.7	11.2	0.3	1.3	9.0	0.3
Mean	3.9	7.7	0.2	1.1	5.9	0.2
St. Deviation	1.1	1.8	0.04	1.1	1.5	0.05

Table 2. Criteria for P status (mg kg-1) determined by different procedures

Methods	Low	Medium	High/Adequate
AB-DTPA ext. P	< 3.0	4-7	> 7
Olsen P	<10	10-15	>15

Source: Rashid et al. 1996.

Table 3. Value of R showing the correlation of P extraction procedures with each other and plant phosphorus

	WS	Paste P	Olsen P	Resin P	Plant P
AB-DTPA P	0.59	0.15	0.54	0.60	0.47
WS P	1.00	-0.07	0.34	0.29	0.29
Paste P	-	1.00	0.12	0.12	0.21
Olsen P	-	-	1.00	0.54	0.31
Resin P	-	-	-	-	0.70

Crop growth stages and increased nutrient uptake can deplete the soil's nutrient content. Phosphorus (P) applications of 90, 135, and 180 kg ha^{-1} resulted in considerably greater extractable P concentrations than the control and 45 kg P2O5 ha-1 at all phases of crop development. However, there were no significant differences between the three higher P levels. This lack of significant variation at higher P levels may Regardless of the crop's growth stage or phosphorus (P) levels, the AB-DTPA extractable P deficiency persisted, resulting in values below 3.0 mg kg^{-1} when 180 kg P2O5 ha^{-1} was administered (Rashid et al., 1988; Sharif, 1985). When phosphorus is added to highly calcareous soil, it transforms into phases that cannot be extracted (Khattak, 1996; Lindsay, 1979).

Extraction of P through Olsen

The soil samples analyzed revealed a range of Olsen P levels from 4.2 to 11.2 mg kg^{-1}, averaging 7.7 mg kg^{-1}. The narrow variation in Olsen P values (standard deviation of 1.8) indicated consistent phosphorus deficiency across the majority of the 20 samples. 80% of the samples were deficient in P, while the remaining 20% showed medium P levels. No soil samples exhibited P toxicity or adequate P levels. This P deficiency suggests a potential limitation on plant growth and development. To optimize agricultural productivity and nutrient uptake, addressing this issue through appropriate fertilization strategies is essential. Notably, excessive phosphorus application was not observed in any soil sample. However, closely monitoring P levels and adjusting fertilization practices is crucial to avoid potential environmental issues related to phosphorus runoff. In Mediterranean environments, (Matar et al., 1992) observed that Olsen P threshold values increased with greater aridity. This phenomenon was explained by the fact that P primarily moves to the roots through diffusion in the soil solution, which is hindered when the soil is dry. Additionally, reduced soil water content leads to higher ionic strength in the soil solution. This promotes P adsorption in soils when the pH exceeds a certain threshold, affecting the equilibrium between solid and water phases and decreasing P release from sorbent surfaces. Therefore, maintaining consistent soil moisture improves P utilization by crops, resulting in lower threshold values for fertilizer response. It is crucial to account for climatic conditions in each growing season.

Water-Soluble P in Paste and Suspension Extract

The water-soluble phosphorus content in the soil ranged from 0.7 to 1.3 mg kg^{-1} (mean: 1.1 mg kg^{-1}) with a small variation. The paste phosphorus content ranged from 0.1 to 0.3 mg L^{-1} (mean: 0.2 mg L^{-1}), also showing a narrow range of variation. Overall, both the water-soluble and paste phosphorus contents demonstrate consistent concentrations, indicating a stable and homogeneous distribution of phosphorus in the soil sample. Lata Verma and Marschner, (2013) stated that the decrease in water-soluble P in the soil at a later stage of incubation may be a result of the assimilation of readily available P (WSP) by microbes. Similar outcomes were observed in soils incubated with rock phosphate and organic matter for 75 days, where water-soluble P levels decreased significantly. Overall, the level of accessible phosphorus (P) initially decreased over the course of 28 days and then gradually rose during a later phase of the incubation period. This suggests that compost with RP charge releases P at a slow rate but over an extended duration. The decline in available P could be attributed to its temporary conversion into an unavailable form. Results like those reported by Bangar et al. (1985); Biswas and Narayanasamy, (2006) were seen after applying phosphorus-rich compost to soil.

Resin P

Resin P levels varied greatly amongst the sites tested, with concentrations ranging from 3.9 to 9.0 mg L^{-1} (on average, 5.0 mg L^{-1}) of phosphorus. The variance in Resin P values was rather small, but it was still there, and it could have an impact on plant growth and nutrient availability. Resin P concentrations show a substantial amount of fluctuation, as indicated by a standard deviation value of 1.5. Soil type, land use practices, and environmental factors all have a role in the observed variation. Knowing this variance is critical for efficient soil management and nutrient planning since it paves the way for individualized fertilization techniques and precise interventions. A high association (R2 = 0.86) was found between NaHCO3-P and phosphorus uptake in ryegrass grown in pots (Sibbesen, 1978). For algae, it was an accurate indicator of phosphorus availability (Zhou et al., 2001). Ellis and Stanford (1988) discovered a strong (p 0.05) correlation between NaOH-P and NTA-P and the phosphorus availability in selenastrum. Fe oxide paper-P showed a strong association with the growth of Anabaena, Euglena,

Selena strum, and Ankistrodesmus ($p < 0.001$) (Sharpley, 1993). Anion resin-P also exhibited a high correlation (R2 = 0.90) with P uptake in pot-grown ryegrass (Sibbesen, 1978), simulating the decrease of P at the surface of freshwater (Uusitalo et al., 2000).

P Concentration in plants Plant P concentrations in the dataset have an average value of 0.2%, indicating a relatively low concentration overall. The range of concentrations spans from 0.1% to 0.3%, suggesting variability but within a narrow range. A low standard deviation of 0.05 confirms the narrow range and indicates consistency and stability in the dataset. To understand the observed variation better, additional analysis and experiments could explore factors such as environmental conditions, nutrient availability, genetic variations, and other variables impacting P uptake and utilization by plants.

The Concentration of Plant P with Soil P Extracted By Different Methods

The correlations between different procedures for soil P extraction were examined. AB-DTPA showed significant correlations with Water-soluble P (R = 0.59), Olsen P (R = 0.54), and Resin P (R = 0.60), but had weak correlations with P determined in paste extract. However, the positive correlations observed in AB-DTPA were consistent with other procedures except for paste P. Paste P had low levels that couldn't be accurately determined and varied greatly among soil samples, unlike other procedures. water-soluble P did not show significant correlations with any other extraction method. Paste P also didn't show significant correlations with any method. Olsen P only showed significance with Resin P. These findings indicate that the correlations between methods depend on soil types, fertilizer application history, total phosphorus content in the soil, and moisture content during sampling. (Table .3) Resin P demonstrated the strongest correlation with plant P ($r^2 = 0.484$), followed by AB-DTPA extractable P ($r^2 = 0.217$). These correlations are considered significant as the R-value exceeds 0.5. Other soil P extraction methods, such as water-soluble P ($r^2 = 0.083$), Olsen P ($r^2 = 0.098$), and Paste P ($r^2 = 0.043$), exhibited weaker and less significant correlations with plant P.(Figure 1 -5) showed the graphical presentation of different values of correlation of plant P with water-soluble P, AB-DTPA phosphorus, Olsen P, Resin P and soil paste P. The resin P and AB-DTPA methods were found to be the most suitable for determining phosphorus (P) levels in the tested soil and plant conditions. Different soil characteristics can influence the variability of these results, as observed by other researchers. For example, the correlation between total plant-available P and Olsen P was not significant, indicating that Olsen P is not a reliable indicator for managing fertilizer in the tested soils. In non-calcareous soils, various methods used to estimate available P showed significant correlations with plant uptake, particularly the Morgan and water-soluble estimates. In calcareous soils, the recommended method is AB-DTPA extraction. AB-DTPA extractable P levels showed a highly significant correlation with plant uptake of P. Compared to other methods like Colwell or resin, DGT (diffusive gradients in thin films) showed a better assessment of plant-available P concentrations in soil based on previous studies.

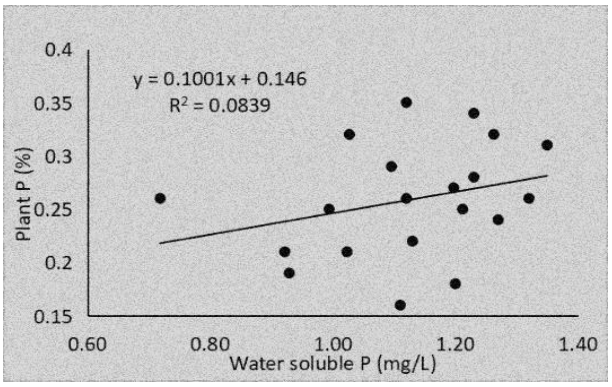

Figure 1 Correlation of Water-soluble P with plant P

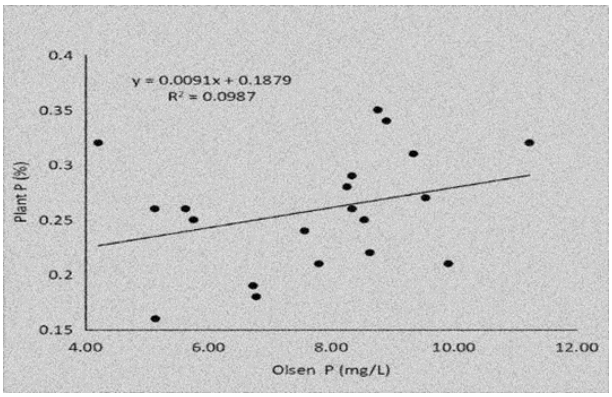

Figure 2 Correlation of AB-DTPA ext. P and plant P

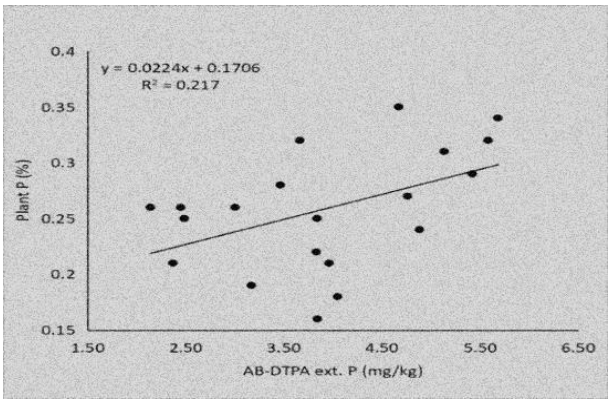

Figure 3 Correlation of Olsen P with plant P

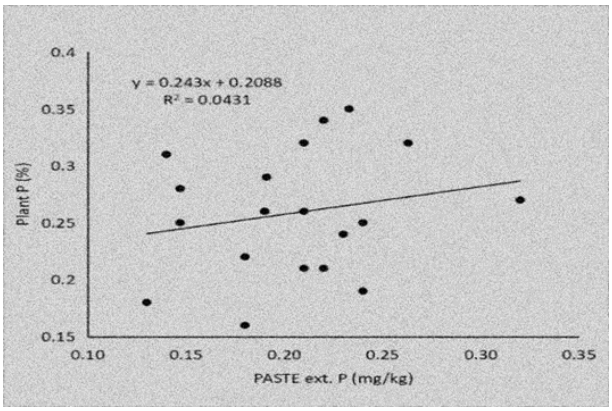

Figure 4 Correlation of Paste ext. P with and plant

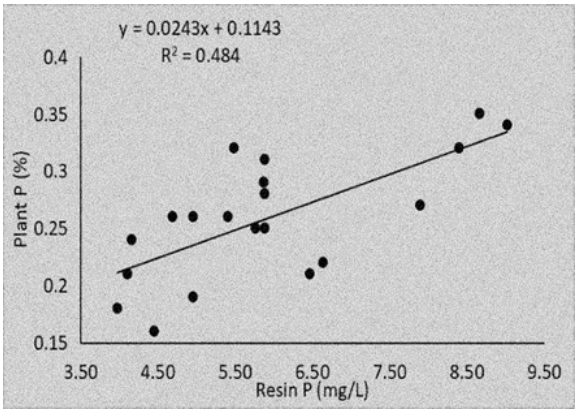

Figure 5 Correlation of Resin P with Plant P

Conclusions and Recommendations

The results of soil phosphorus extraction methods varied among the samples. AB-DTPA extractable phosphorus was deficient in 20% of the samples, while the Olsen method indicated low soil phosphorus levels in 80% of the samples. AB-DTPA phosphorus correlated strongly with water-soluble phosphorus, Olsen phosphorus, and Resin phosphorus but weakly with paste extract phosphorus. Water-soluble and paste extract phosphorus did not show significant correlations with any method, while Olsen phosphorus correlated significantly with Resin phosphorus. Resin phosphorus had the highest correlation with plant phosphorus, followed by AB-DTPA extractable phosphorus, indicating that these two methods are most suitable for phosphorus determination in the given soil and plant conditions. The recommended methods for determining and recommending fertilizer for phosphorus (P) in the soil and climate conditions are AB-DTPA ext. P and Resin P, as they show significant correlations with plant P, indicating its availability to plants. Further research should be conducted in larger areas and various agroecological zones to optimize fertilizer recommendations for different crops and soils.

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Conflict of interest

We declare no conflict of interest.

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Effects of 5-Aminolevulinic Acid (5-ALA) on Morphological and Physiological Characteristics of Grapevine against Salt Stress

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ABSTRACT

Salinity, one of the most significant abiotic stress factors restricting plant production, causes the destruction of agricultural lands and reduces productivity. In recent years, the utilization of 5-aminolevulinic acid (5-ALA) applications, which have important effects in terms of avoiding and providing tolerance to factors by impacting the physiology and metabolism of the plants, has been on the agenda. In this research, the impacts of foliar treatments of different levels of 5-ALA (0, 0.3, 0.6 and 0.9 mM) on morphological and physiological traits of 41 B American grapevine rootstocks under salinity stress (NaCl solution starting with 25 mM and reaching 150 mM concentration) were investigated. Salinity stress caused significant decreases in growth parameters, chlorophyll content, RWC and stomatal conductance, and significant increases in leaf temperature, proline and MDA content, physical damage and membrane damage degree. Under salinity stress, 0.9 mM 5-ALA treatments resulted in significant increases in shoot length (14.67 cm), root length (34.50 cm), leaf thickness (0.23 µm) leaf area (31.37 cm^2), leaf number (8.67 pieces), chlorophyll content (21.83 SPAD), RWC (80.20%), proline content (0.19 $\mu mol.g^{-1}$) and stomatal conductance (78.05 $mmol.m^{-2}.s^{-1}$); and significant decreases in physical damage degree (1.00 scale degree), membrane injury degree (15.46%) and MDA content (28.20 $nmol.g^{-1}$) compared to non-ALA treatments. According to the results of this study, 5-ALA can be recommended as an alternative application to provide salinity tolerance in plants in order to reduce the damage caused by salinity stress in agricultural lands.

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Introduction

Salinity, which is among the most significant abiotic stressors restricting plant production, causes the destruction of agricultural lands, reduces productivity and leads to significant economic losses (Qadir et al., 2014). Viticulture, with a global output of 77.4 million tons and an area of 7.3 million hectares, is among the major agricultural sectors that will be impacted by salinity damage.

On a global scale, it is estimated that more than 930 million hectares of land are confronted with the issue of soil salinity (Gregory et al., 2018). Due to climate change, increased evapotranspiration is predicted to accelerate soil salinization (Phogat et al., 2020). By 2050, there is a risk that 25-73% of existing vineyard areas under the Mediterranean climate will cease to be suitable for cultivation as a result of land desertification due to salinity and associated water scarcity (Hannah et al., 2013). Reports from leading grape-producing countries indicate that salinity is of particular concern for viticulture in some regions in Greece, India, Turkey, Italy, Australia, Iran, the US and Spain (Baneh et al., 2014; Phogat et al., 2020). In the Australian context, the prospective overall advantage of improving both soil salinity and sodicity was calculated to be $42 million annually for grape cultivation in 2005. This figure constitutes approximately 13% of the average production profit (Hajkowicz & Young, 2005).

The challenge of salinity, exacerbated particularly during hot and arid periods, typically arises due to inadequate rainfall, elevated evapotranspiration rates, or the utilization of irrigation water containing high concentrations of Na^+ and Cl^-. This leads to an escalation in salt concentration within the root zone (Tate, 2001; Hannah et al., 2013). Excess salt in the soil causes a number of metabolic disturbances in plants, particularly as a consequence of osmotic influences (dehydration), nutritional disorders and Na^+ toxicity (Munns, 2002). The early stage of the growth reaction in the presence of salt stress exhibits characteristics similar to those displayed by

plants experiencing water scarcity, attributed to disruptions in osmotic balance (Munns, 2002). Salt stress leads to growth inhibition, reduced photosynthetic activity and membrane stability, accumulation of specific osmolytes in tissues and induction of oxidative stress (Kozminska et al., 2018). As a result, Na^+ and Cl^- buildup on leaves and roots, as well as the induction of malondialdehyde (MDA), glutathione reductase (GR), superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) activities, and an increase in total phenolics and flavonoids are known to occur (Kumar et al., 2017). Salinity also significantly affects the yield and quality of plants through changes in cellular water content and osmotic potential (Yuanchun et al., 2015).

In the classification by Maas and Hoffman (1977), *Vitis vinifera* is categorized as a species moderately sensitive to salinity, with an upper limit of electrical conductivity (EC) around ~2.0 $dS.m^{-1}$, influencing factors such as fruit set and yield (Baby et al., 2016). In comparison to the majority of other plant species, this value is relatively low, as highlighted by Munns & Tester in 2008. On the flip side, the grapevine is recognized for its resilience to drought conditions (Charrier et al., 2018). Potential effects of high salinity on grapevine growth and productivity include reduced leaf expansion rates, leaf blight or mortality, reduced fruit set and significant yield losses (Munns & Tester, 2008; Baby et al., 2016).

Especially with the climatic changes that are predicted to continue in recent years, there is a need to develop new strategies that can eliminate or minimize the effects of increasing soil salinity on vine growth and development, yield and quality. In this regard, elicitors, which are naturally occurring and produced by organisms and play an important role in alleviating the effects of various stresses on plants, are seen as promising applications. Among the elicitors is 5-Aminolevulinic acid (5-ALA), which affects plant physiology and metabolism to prevent and tolerate stress factors. Acting as an important phytohormone that governs plant growth and development, 5-ALA functions as an environmentally friendly, biodegradable and non-toxic plant growth regulator (Xiong et al., 2020; Yang et al., 2021).

Studies have shown that exogenous 5-ALA applications are an effective strategy for increasing plant tolerance to different environmental stress factors, including salinity. The effectiveness of 5-ALA in enhancing salinity tolerance shows variation among different plant species, and the concentration range for optimal effectiveness also differs across these species. However, studies to evaluate the effects of 5-ALA on the defense mechanisms of grapevines under salt stress are quite limited. Especially in American grapevine rootstocks, which constitute the subsoil parts of the grapevine and will be primarily affected by salt stress conditions, no study has been found to examine the effects of 5-ALA applications. Therefore, there is a need to elucidate the mechanisms of 5-ALA applications on the salinity tolerance of grapevine and to determine the most effective 5-ALA concentration.

In this research, the impacts of various concentrations of exogenous 5-ALA foliar applications on morphological, physiological and biochemical parameters of grapevine rootstocks subjected to salt stress were investigated.

Material and Method

Research Area and Plant Material

This experiment, conducted in the greenhouse and research laboratories of Yozgat Bozok University Faculty of Agriculture between 2022 and 2023, aimed to explore the effects of different concentrations of 5-ALA applications on grapevine rootstocks under salinity stress.

In the study, 1-year old cuttings of the *V. vinifera* × *V. berlandieri* hybrid 41 B (41 B Millardet et de Grasset, 41 B MGt) American grapevine rootstock, which is characterized by its sensitivity to salinity (Çelik, 1996), although it shows a very high resistance to lime in cultivation, and due to this feature, damage symptoms due to salinity stress can be observed significantly, were used as plant material.

Preparation of Growing Media, Planting of Cuttings and Cultivation of Plants

Prior to planting, 41 B American grapevine rootstocks were subjected to bud removal (a single bud was left) and bottom freshening. Rootstock cuttings were subjected to rapid dipping treatment with IBA (Indole Butyric Acid) at a concentration of 2000 ppm and then planted in 15×15×18 cm black PE pots made up of an equally large volume of sterilized peat and perlite. The cuttings were promptly watered following transplantation, and irrigation persisted until water began to drain out from the pot's drainage holes.

The research area where the plants were grown is a ~200 m^2 greenhouse with a spring roof, polycarbonate material, 70% shade screen, fan heater, fan & pad system and ventilation system with a concrete floor. In the greenhouse where the experiment will be established, there are rooting tables 5 m long, 1.20 m wide, 80 cm above the ground and 20 cm deep. The pots in which the cuttings were planted were placed on these tables.

5-ALA and Salinity Stress Applications

The study utilized 5-Aminolevulinic acid hydrochloride (CAS No: 5451-09-2) from the SIGMA company as the source of 5-ALA. Saplings at phenological stage 12-15 (shoot lengths of 10-15 cm) according to the modified Eichhorn-Lorenz (E-L) system introduced by Coombe (1995) were used in the experiment and 5-ALA solutions at concentrations of 0, 0.3, 0.6 and 0.9 mM were sprayed on the entire green surface of the plants ~6 weeks after planting.. Four weeks after 5-ALA applications, the growing media were irrigated with NaCl solution, which was started with 25 mM and increased by 25 mM weekly to 150 mM concentration. Purified water was used in control samples.

After a 120-day growing period in which adequate root and shoot development was achieved, the experiment was terminated and morphological, physiological and biochemical characteristics of the grapevine saplings were analysed.

The Effects of 5-ALA on Plant Growth Parameters

Shoot and root fresh weights were weighed using an analytical balance and the averages were expressed in g. The dry weights of shoots and roots were weighed using an analytical balance after drying in an air-circulating oven at 65°C for 72 hours and the averages were expressed in mg.

Shoot and root lengths were determined by measuring the distances from the tip to the base in cm using a ruler.

Leaf surface area was measured from mature leaves using an area meter (ADC BioScientific Area Meter AM 300) and the mean values were recorded in cm^2.

Leaf thickness was determined by mechanical micrometer (BTS-12051) and values were expressed in µm.

The degree of physical damage was determined using the scale (0-3 scale) developed by Sivritepe & Eriş (1999). Accordingly, those with no necrotic tissues on shoots and leaves caused by salinity stress were scored as "grade 0", those with necrosis on shoot tips and leaf margins were scored as "grade 1", those with necrosis on more than 50% of the leaf and/or part of the shoot were scored as "grade 2", and those with necrosis causing plant death were scored as "grade 3".

The Effects of 5-ALA on Physiological Characteristics

Chlorophyll content was assessed using a handheld chlorophyll meter (Konica Minolta SPAD 502) by measuring between the veins of the leaves. The values measured were represented in SPAD (Geravandi et al., 2011).

The relative water content of leaves was determined following the method outlined by Yamasaki & Dillenburg (1999). Accordingly, the fresh weight (FW) of the leaves was first determined. The leaves were immersed in distilled water for a duration of 6 hours, and their turgor weights (TW) were subsequently measured. Following this, the dry weights (DW) were determined by subjecting the leaves to 80°C for 24 hours. The relative water content (%) was calculated using the formula [(FW-DW)/(TW-DW)]×100.

Leaf temperature and stomatal conductance were measured between the veins of the leaves using a leaf porometer (Decagon/Pullman, WA, SC-1 Leaf Porometer) and recorded in $mmol.m^{-2}.s^{-1}$ and °C, respectively.

The membrane damage degree was computed by measuring the electrolyte removed from the cell. For this purpose, 3 discs of 6 mm in diameter were first removed from the leaves with the help of cork-borer. These discs were soaked in 20 ml distilled deionized water for four hours and EC1 was measured using an EC meter (Jenway-470 condimeter). After the same discs were kept at 100 °C for 10 min, EC_2 was measured and calculated as percentage (%) with the formula $(EC_1/EC_2)\times100$ (Nayyar, 2003).

The Effects of 5-ALA on Biochemical Characteristics

Lipid peroxidation was assessed by quantifying malondialdehyde (MDA) using the methodology outlined in the procedure by Lutts et al. (1996). MDA was measured by reading the color developing at 535 nm and 600 nm and the values were recorded as $nmol.g^{-1}$.

Proline was determined spectrophotometrically (Lambda 25, Perkin Elmer) at 520 nm using the ninhydrin assay according to the procedure of Bates et al. (1973) and the results were recorded as $\mu mol.g^{-1}$.

Experimental Design and Evaluation of Data

The study was designed based on the randomized plots trial design with three replicates and each replicate consisted of 20 plants. The numerical data obtained were processed using IBM SPSS 20.0 program. Analysis of variance (One-Way ANOVA) was applied to the data. The Duncan multiple comparison test (with a significance level of $p<0.05$) was employed to assess the distinctions among the means.

Results

The Effect of 5-ALA on Plant Growth Parametersand Chlorophyll Content

Salinity stress caused statistically significant decreases ($p<0.05$) in shoot length of grapevine saplings (16.33 cm) compared to non-stressed groups (7.83 cm). However, there was no significant difference in shoot fresh and dry weight between salinity stress and non-salinity stress groups. 5-ALA treatments resulted in statistically significant ($p<0.05$) increases in shoot length in both salinity stressed and non-salinity stressed groups compared to non-ALA treatments. In terms of shoot length under salinity stress, 0.6 and 0.9 mM 5-ALA treatments showed higher averages (12.50 cm and 14.67 cm, respectively) compared to the negative control (7.83 cm). In the non-salt-stressed groups, 0.9 mM 5-ALA treatment had a higher value in shoot length (28.17 cm) compared to the positive control (16.33 cm) (Figure 1) (Table 1).

Salinity stress caused statistically significant decreases ($p<0.05$) in root length of grapevine saplings (19.17 cm) compared to non-stressed groups (34.33 cm). However, there was no significant difference in root fresh and dry weight between salinity stress and non-salinity stress groups. In both salinity stressed and non-salinity stressed groups, 5-ALA treatments resulted in statistically significant increases in root length compared to non- 5-ALA treatments ($p<0.05$). All 5-ALA treatments under salinity stress resulted in significant increases in root length, especially 0.9 mM 5-ALA treatment had higher averages (34.50 cm) compared to other concentrations and negative control. In the groups without salinity stress, 0.9 mM 5-ALA application had higher values in terms of root length (59.00 cm) compared to the positive control (Figure 2) (Table 2).

Table 1. Effects of 5-ALA on shoot traits

Treatments	Shoot Fresh Weight (g)	Shoot Dry Weight (mg)	Shoot Length (cm)
Negative Control	2.81±2.03	0.87±0.60	7.83±2.84 f
Positive Control	3.36±0.65	1.08±0.28	16.33±1.26 bd
0.3 mM 5-ALA+NaCl	3.58±1.02	1.09±0.29	11.00±1.32 ef
0.3 mM 5-ALA	4.27±0.67	1.31±0.22	18.33±0.76 bc
0.6 mM 5-ALA+NaCl	3.39±1.04	1.03±0.32	12.50±0.50 de
0.6 mM 5-ALA	4.53±0.86	1.47±0.21	20.17±0.76 b
0.9 mM 5-ALA+NaCl	6.15±2.85	1.93±0.97	14.67±0.29 ce
0.9 mM 5-ALA	3.27±0.79	1.02±0.27	28.17±5.48 a
Mean	3.92±1.57	1.22±0.50	16.13±6.31

*Different letters indicate significant differences based on Duncan's post-hoc analysis at $p \leq 0.05$.

Table 2. Effects of 5-ALA on root traits

Treatments	Root Fresh Weight (g)	Root Dry Weight (mg)	Root length (cm)
Negative Control	6.54±0.78	3.34±1.09	19.17±3.33 f
Positive Control	5.50±2.05	2.27±1.39	34.33±0.58 cd
0.3 mM 5-ALA+NaCl	3.43±1.91	1.58±0.81	27.00±1.00 e
0.3 mM 5-ALA	4.96±1.94	1.85±0.39	36.83±1.89 c
0.6 mM 5-ALA+NaCl	6.08±2.82	2.43±1.27	30.67±2.31 de
0.6 mM 5-ALA	4.88±2.60	1.94±0.88	47.67±6.81 b
0.9 mM 5-ALA+NaCl	2.05±0.30	0.94±0.14	34.50±0.00 cd
0.9 mM 5-ALA	2.16±1.16	1.16±0.30	59.00±2.65 a
Mean	4.45±2.27	1.94±1.04	36.15±12.05

*Different letters indicate significant differences based on Duncan's post-hoc analysis at p ≤0.05.

Table 3. Effects of 5-ALA on leaf characteristics and chlorophyll content

Treatments	Leaf Thickness (µm)	Leaf Area (cm^2)	Number of Leaves (piece)	Chlorophyll Content (SPAD)
Negative Control	0.17±0.01 c	19.70±2.52 e	6.33±1.15 d	17.90±2.26 c
Positive Control	0.15±0.01 c	31.64±1.54 c	8.67±0.58 bc	22.60±1.31 ab
0.3 mM 5-ALA+NaCl	0.19±0.02 b	25.25±1.97 d	7.67±0.58 cd	20.33±0.67 bc
0.3 mM 5-ALA	0.15±0.01 c	36.32±0.90 b	8.00±1.00 bc	22.93±2.01 ab
0.6 mM 5-ALA+NaCl	0.21±0.01 a	27.23±2.20 d	7.67±0.58 cd	20.73±1.55 bc
0.6 mM 5-ALA	0.15±0.01 c	39.48±2.20 ab	9.33±0.58 ab	22.87±0.64 ab
0.9 mM 5-ALA+NaCl	0.23±0.01 a	31.37±2.95 c	8.67±0.58 bc	21.83±1.55 ab
0.9 mM 5-ALA	0.15±0.01 c	41.74±1.61 a	10.33±0.58 a	23.93±1.99 a
Mean	0.18±0.03	31.59±7.32	8.33±1.31	21.64±2.27

*Different letters indicate significant differences based on Duncan's post-hoc analysis at p ≤0.05.

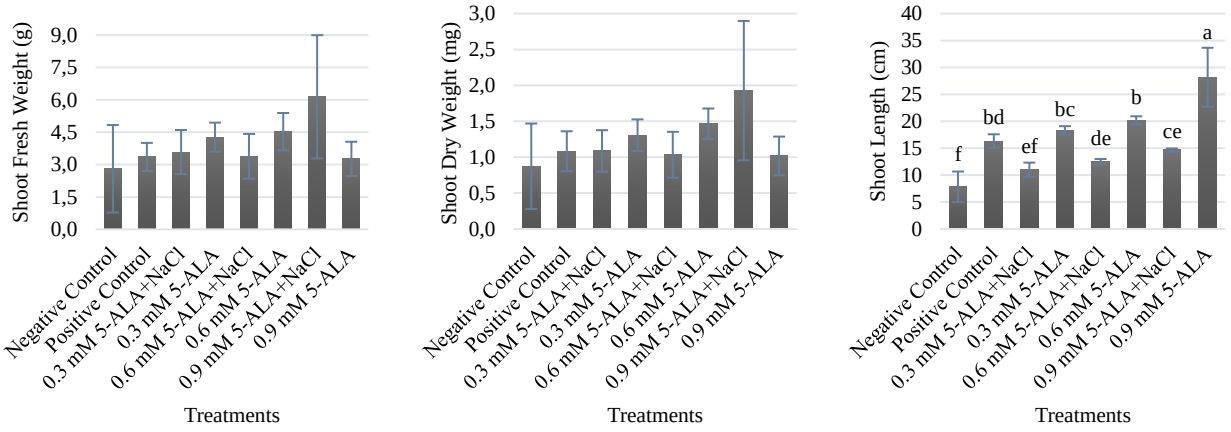

Figure 1. Effects of 5-ALA on shoot traits. Different letters indicate significant differences based on Duncan's post-hoc analysis at p ≤0.05.

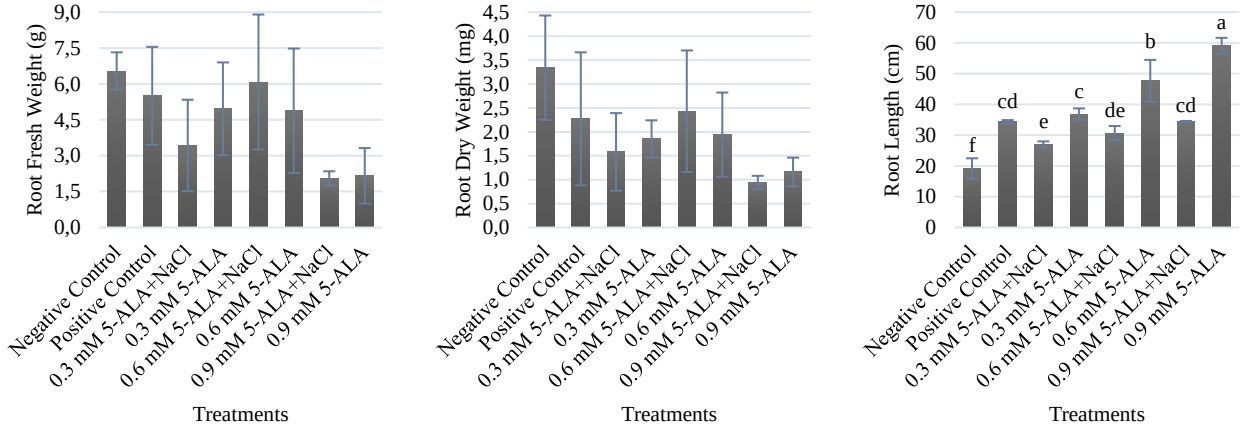

Figure 2. Effects of 5-ALA on root traits. Different letters indicate significant differences based on Duncan's post-hoc analysis at p ≤0.05.

Salinity stress caused statistically significant decreases (19.70 cm^2, 6.33 pieces and 17.90 SPAD, respectively) in leaf area, leaf number and chlorophyll content of grapevine saplings (31.64 cm^2, 8.67 pieces and 22.60 SPAD, respectively) compared to non-stressed groups ($p<0.05$). Nevertheless, there was no notable alteration in leaf thickness observed between the positive and negative control groups. In both salinity stressed and non-salinity stressed groups, 5-ALA treatments resulted in statistically significant increases in leaf area and leaf number compared to non-ALA treatments ($p<0.05$). While all 5-ALA treatments under salinity stress provided significant increases in leaf thickness and leaf area, the most effective concentration was 0.9 mM 5-ALA treatment (0.23 µm and 31.37 cm^2, respectively) compared to the negative control (0.17 µm and 19.70 cm^2, respectively) ($p<0.05$). In terms of leaf number and chlorophyll content, only 0.9 mM 5-ALA treatment caused a statistically significant increase (8.67 pieces and 21.83 SPAD, respectively) compared to the negative control (6.33 pieces and 17.90 SPAD, respectively) ($p<0.05$). All 5-ALA treatments (0.3 mM 5-ALA: 36.32 cm^2; 0.6 mM 5-ALA: 39.48 cm^2; 0.9 mM 5-ALA: 41.74 cm^2) provided a statistically significant increase in leaf area compared to the positive control (31.64 cm^2) in the groups without salinity stress. In terms of leaf number, 0.9 mM 5-ALA treatment (10.33 pieces) had higher values compared to the positive control (8.67 pieces), while there was no statistically significant change in leaf thickness or chlorophyll content (Figure 3) (Table 3).

The Effect of 5-ALA on Stomatal Conductivity, Leaf Temperature, Proline Content and Leaf Relative Water Content

Salinity stress caused statistically significant decreases (59.04% and 72.38 $mmol.m^{-2}.s^{-1}$, respectively) in RWC and stomatal conductance of grapevine saplings (82.30% and 81.29 $mmol.m^{-2}.s^{-1}$, respectively) compared to non-stressed groups ($p<0.05$). However, leaf temperature and proline content increased significantly (22.33 °C and 0.13 $\mu mol.g^{-1}$, respectively) compared to non-stressed groups (21.40 °C and 0.08 $\mu mol.g^{-1}$, respectively) ($p<0.05$). In both salinity-stressed and non-salinity-stressed groups, 5-ALA treatments resulted in statistically significant increases in RWC ratio, stomatal conductance and proline content compared to non-5-ALA treatments ($p<0.05$). All 5-ALA treatments under salinity stress resulted in significant increases in RWC ratio (0.3 mM 5-ALA: 68.45%; 0.6 mM 5-ALA: 74.68%; 0.9 mM 5-ALA: 80.20%) compared to the negative control (59.04%). However, 0.6 and 0.9 mM 5-ALA treatments caused a statistically significant increase in proline content (0.16 and 0.19 $\mu mol.g^{-1}$, respectively) compared to the negative control (0.13 $\mu mol.g^{-1}$). In terms of stomatal conductance, 0.9 mM 5-ALA treatment caused a statistically significant increase (78.05 $mmol.m^{-2}.s^{-1}$) compared to the negative control (72.38 $mmol.m^{-2}.s^{-1}$). Nevertheless, there was no statistically significant alteration observed in leaf temperature. In the groups without salinity stress, 0.6 and 0.9 mM 5-ALA treatments had higher values for stomatal conductance (86.30 and 86.80 $mmol.m^{-2}.s^{-1}$, respectively) compared to the positive control (81.29 $mmol.m^{-2}.s^{-1}$). However, for RWC and proline content, 0.9 mM 5-ALA treatment showed higher values (93.48% and 0.11 $\mu mol.g^{-1}$, respectively) compared to the positive control (82.30% and 0.08 $\mu mol.g^{-1}$, respectively). In terms of leaf temperature parameter, 0.9 mM 5-ALA treatment had lower mean values (20.49°C) compared to the positive control (21.40°C) ($p<0.05$) (Figure 4) (Table 4).

Table 4. Effects of 5-ALA on RWC, stomatal conductance, leaf temperature and proline content

Treatments	RWC (%)	Stomatal Conductivity ($mmol.m^{-2}.sn^{-1}$)	Leaf Temperature (°C)	Proline Content ($\mu mol.g^{-1}$)
Negative Control	59.04±9.49 e	72.38±2.21 e	22.33±0.29 a	0.13±0.01 c
Positive Control	82.30±0.83 bc	81.29±1.87 bc	21.40±0.44 bc	0.08±0.01 e
0.3 mM 5-ALA+NaCl	68.45±2.36 d	75.19±1.42 de	22.33±0.29 a	0.14±0.01 c
0.3 mM 5-ALA	83.59±1.05 b	84.10±3.41 ab	20.70±0.57 cd	0.09±0.01 e
0.6 mM 5-ALA+NaCl	74.68±4.56 cd	76.20±2.62 de	21.90±0.44 ab	0.16±0.01 b
0.6 mM 5-ALA	85.48±2.86 b	86.30±3.63 a	20.69±0.53 cd	0.09±0.02 de
0.9 mM 5-ALA+NaCl	80.20±4.82 bc	78.05±1.24 cd	21.59±0.55 ab	0.19±0.02 a
0.9 mM 5-ALA	93.48±2.09 a	86.80±3.68 a	20.49±0.53 d	0.11±0.01 d
Mean	78.40±10.91	80.04±5.62	21.43±0.81	0.12±0.04

*Different letters indicate significant differences based on Duncan's post-hoc analysis at $p \leq 0.05$.

Table 5. Effects of 5-ALA on oxidative stress parameters

Treatments	Physical Damage Degree (0-3 scale)	Membrane Damage Degree (%)	MDA ($nmol.g^{-1}$)
Negative Control	3.00±0.00 a	17.94±0.29 a	37.25±0.33 a
Positive Control	0.00±0.00 d	14.83±0.74 d	16.85±0.18 e
0.3 mM 5-ALA+NaCl	2.00±0.00 b	17.00±0.82 ab	31.39±1.50 b
0.3 mM 5-ALA	0.00±0.00 d	14.80±0.70 d	22.39±1.33 d
0.6 mM 5-ALA+NaCl	1.00±0.00 c	16.24±0.31 bc	32.32±0.22 b
0.6 mM 5-ALA	0.00±0.00 d	14.38±0.32 d	21.49±0.64 d
0.9 mM 5-ALA+NaCl	1.00±0.00 c	15.46±0.88 cd	28.20±1.39 c
0.9 mM 5-ALA	0.00±0.00 d	12.85±0.25 e	21.29±0.52 d
Mean	0.88±1.08	15.44±1.61	26.40±6.69

*Different letters indicate significant differences based on Duncan's post-hoc analysis at $p \leq 0.05$.

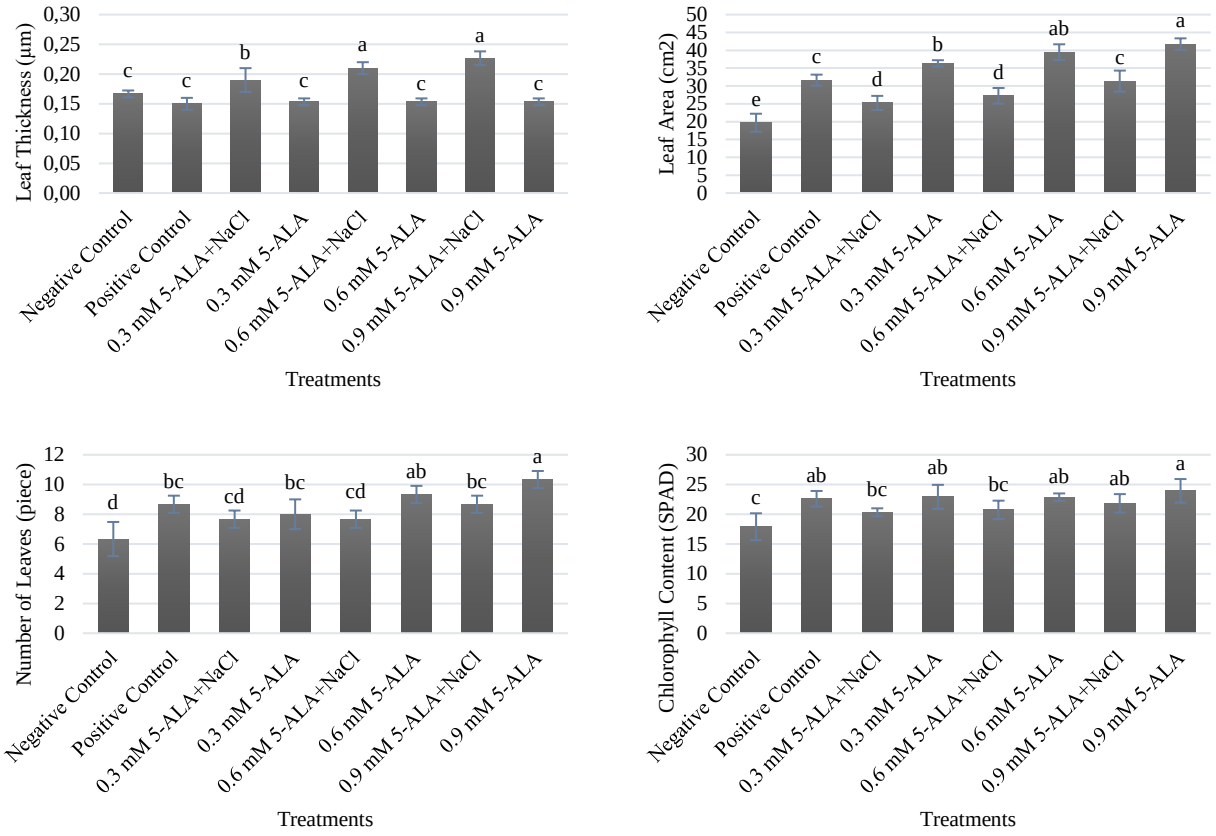

Figure 3. Effects of 5-ALA on leaf characteristics and chlorophyll content. Different letters indicate significant differences based on Duncan's post-hoc analysis at p ≤0.05.

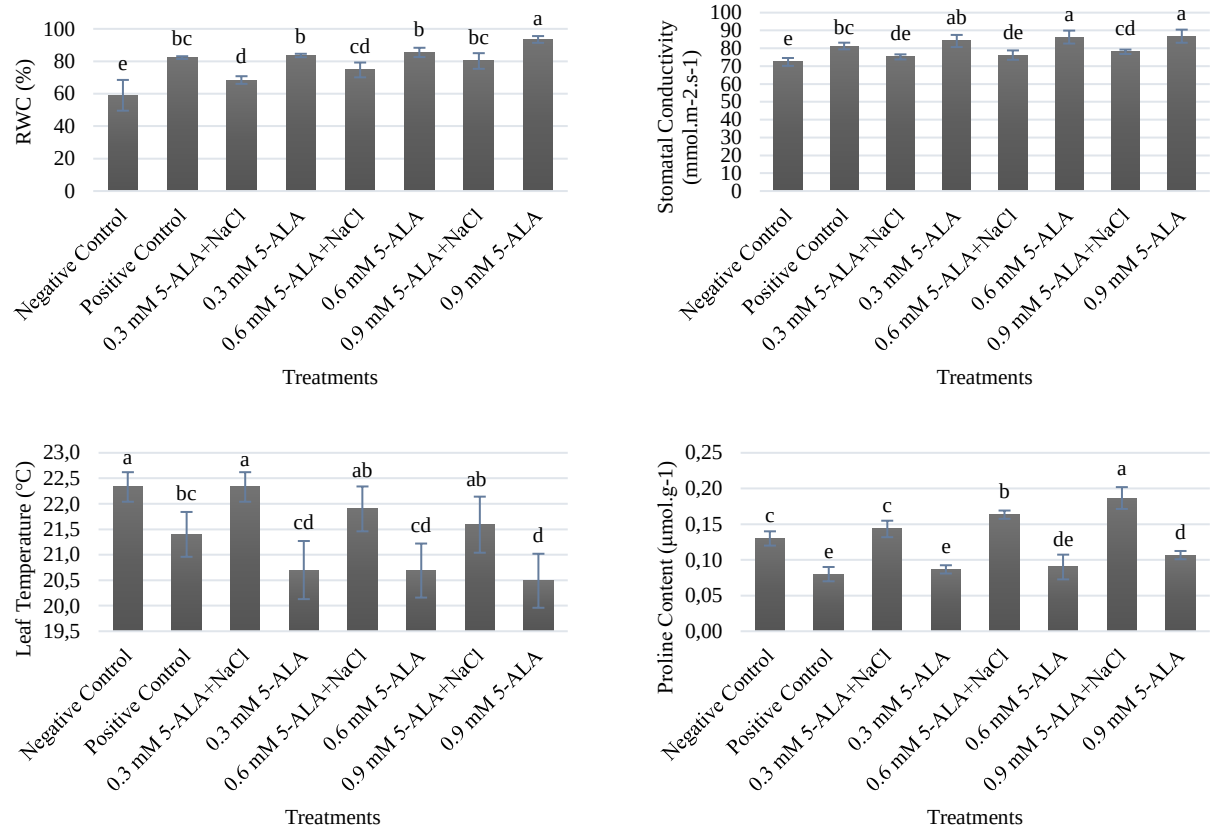

Figure 4. Effects of 5-ALA on RWC, stomatal conductance, leaf temperature and proline content. Different letters indicate significant differences based on Duncan's post-hoc analysis at p ≤0.05.

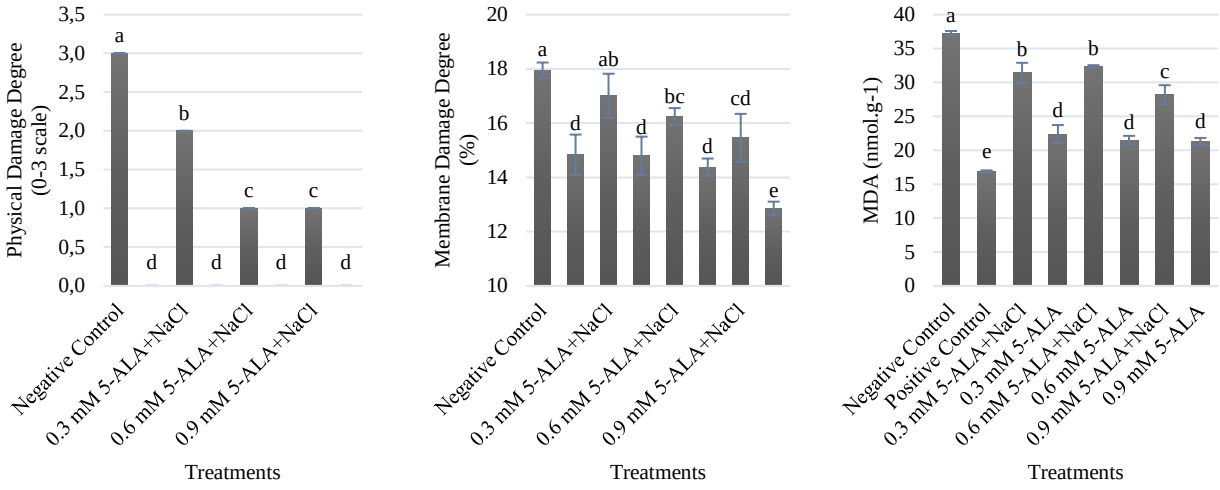

Figure 5. Effects of 5-ALA on oxidative stress parameters. Different letters indicate significant differences based on Duncan's post-hoc analysis at $p \leq 0.05$.

The Effect of 5-ALA on Oxidative Stress Parameters

Salinity stress caused statistically significant increases (3.00 scale degree, 17.94% and 37.25 nmol.g^{-1}, respectively) in the degree of physical damage, membrane damage and MDA content of grapevine saplings (0.00 scale degree, 14.83% and 16.85 nmol.g^{-1}, respectively) compared to non-stressed groups ($p<0.05$). All 5-ALA treatments under salinity stress showed significant decreases (0.3 mM 5-ALA: 2.00 scale degree and 31.39 nmol.g^{-1}; 0.6 mM 5-ALA: 1.00 scale degree and 32.32 nmol.g^{-1}; 0.9 mM 5-ALA: 1.00 scale degree and 28.20 nmol.g^{-1}) in the degree of physical damage and MDA content compared to the negative control (3.00 scale degree and 37.25 nmol.g^{-1}, respectively) ($p<0.05$). In terms of the degree of membrane damage, 0.6 and 0.9 mM 5-ALA treatments caused a statistically significant decrease (16.24% and 15.46%, respectively) compared to the negative control (17.94%) ($p<0.05$). While 0.9 mM 5-ALA application provided a significant decrease (12.85%) in the degree of membrane damage in the groups without salinity stress compared to the positive control (14.83%), all 5-ALA applications showed a significant increase in MDA content (0.3 mM 5-ALA: 22.39 nmol.g^{-1}; 0.6 mM 5-ALA: 21.49 nmol.g^{-1}; 0.9 mM 5-ALA: 21.29 nmol.g^{-1}) compared to the positive control (16.85 nmol.g^{-1}) ($p<0.05$). No statistically significant difference was found in the degree of physical damage (Figure 5) (Table 5).

Discussion

In the present study, salinity stress caused significant reductions in the growth characteristics (shoot length, leaf area, leaf number and root length) of grapevine saplings compared to non-stressed groups. This growth inhibition in salinity-affected grapevine saplings was thought to be due to osmotic and ionic responses such as oxidative stress, water loss and photoinhibition (Zhou-Tsang et al., 2021). Studies have reported that the osmotic potential of saline soils causes loss of cellular turgor, leading to dehydration of grapevine tissues and consequent growth inhibition, biomass loss and cell death (Munns & Tester 2008; Stevens et al. 2011; Baby et al. 2016).

5-ALA treatments significantly increased growth traits (shoot length, root length, leaf area and number of leaves) in both salinity stressed and non-salinity stressed groups compared to non-ALA treatments. In addition, 5-ALA treatments under salinity stress were effective in increasing leaf thickness. While all 5-ALA concentrations were found to be effective in increasing root length, leaf thickness and leaf area under salinity stress, 0.6 and 0.9 mM 5-ALA treatments were found to be the most effective concentrations in terms of shoot length and 0.9 mM 5-ALA treatment in terms of leaf number. In the groups without salinity stress, all 5-ALA concentrations were found to be effective in terms of leaf area, while 0.9 mM 5-ALA was found to be the most effective concentration in terms of shoot length, root length and number of leaves. Studies have shown that 5-ALA are regulatory substance that promotes plant growth and development under both normal and stressful conditions (Wang et al., 2004; Korkmaz, 2012). Tavallali et al. (2019) reported that foliar application of different concentrations (0, 25 and 50 mg.l^{-1}) of 5-ALA had positive effects on shoot biomass, shoot length, total phenolic content and antioxidative activity in purslane (*Portulaca oleracea* L.); however, the most effective concentration was obtained from 50 mg.l^{-1} concentration. Watanabe et al. (2000) reported that among 12 different plant growth regulators examined against salinity stress in cotton, 5-ALA was the most effective application in terms of increasing plant tolerance. Nishihara et al. (2003) reported that 5-ALA treatments improved growth and increased antioxidative enzyme activity in spinach (*Spinacia oleracea*) under NaCl stress. On the other hand, Yang et al. (2021) reported that leaf size and leaf thickness increased in *Buxus megistopphylla* Levl exposed to various stress factors as a result of foliar spraying of 5-ALA at a concentration of 20 mg.l^{-1}. Manafi et al. (2015) determined that exogenous 5-ALA applications at different concentrations (0, 0.3, 0.6 and 0.9 mM) applied from seed and leaves against cold stress in soybean (*Glycine max* L. Merr) increased plant height, shoot fresh and dry weight and chlorophyll content at 0.3 mM dose. The same researchers found that foliar spray application of 5-ALA was more effective than seed

application. In the present study, it was thought that the positive effect of 5-ALA applications on the growth characteristics of grapevine saplings may be related to the increase in chlorophyll content, photosynthesis and the proportion of enzymatic or non-enzymatic antioxidant systems (Naeem et al., 2010, 2011). Indeed, Hotta et al. (1997a) reported that 5-ALA (10-300 $mg.l^{-1}$) applied at the early growth stage increased growth rate and photosynthesis in different plant species such as paddy, faba bean, barley, potato, radish, garlic and kidney bean. In another study, 5-ALA was found to promote plant growth by increasing the photosynthetic capacity of melon seedlings under low temperatures and low light intensity (Wang et al., 2004). Similar findings were recorded by Xu et al. (2010) in Kudzu (*Pueraria phaseoloides*) and persimmon (*Phoenix dactylifera* L.) and reported that the growth enhancement was related to chlorophyll content and photosynthetic rate.

In the present study, salinity stress caused significant decreases in the chlorophyll content of grapevine saplings compared to the non-stressed groups. Previous studies have shown that chlorophyll fluorescence is attenuated in grapevines under salinity stress due to inhibition of electron transport in photosystem II (Downton, 1983) and this effect has been attributed to ROS-induced peroxidation of membrane lipids (Fozouni et al. 2012).

5-ALA treatments provided significant increases in terms of increasing chlorophyll content under salinity stress compared to 5-ALA untreated groups. The most effective concentration for increasing chlorophyll content under salinity stress was 0.9 mM 5-ALA treatment. In this study, the positive effect of 5-ALA applications on the chlorophyll content of grapevine saplings was evaluated as a result of the fact that 5-ALA constitutes the initial step in the chlorophyll synthesis chain in plants (Scheer, 2004). In a similar study conducted on grapevine, Watanabe et al. (2006) found that the application of 100 $mg.l^{-1}$ 5-ALA increased plant growth and CO_2 assimilation. On the other hand, Hotta et al. (1997b) found that low concentrations (0.06-0.6 $\mu mol.l^{-1}$) of 5-ALA increased chlorophyll content in horseradish (*Armoracia rusticana*) and golden pothos (*Epipremnum aureum*). It has also been reported by various researchers that exogenous 5-ALA applied at low concentrations increases the photosynthetic capacity and yield by increasing the chlorophyll content in leaves (Watanabe et al., 2000; Youssef & Awad, 2008).

Salinity stress caused significant decreases in RWC ratio and stomatal conductance of grapevine saplings compared to non-stressed groups. On the contrary, leaf temperature and proline content showed an opposite trend and increased significantly compared to the non-stressed groups. Responses to salinity consist of changes in plant physiology or biochemistry as a result of damage or plant responses that attempt to prevent or mitigate damage (Munns et al. 2020). Stomatal regulation (usually closure) is a well-known early response to osmotic and/or drought stress (Zhou-Tsang et al., 2021). In this response, Walker et al. (1981) reported that 5-ALA reduced water loss through transpiration, but caused a decrease in photosynthetic activity by limiting CO_2 diffusion through the stomatal pores to the leaf and increasing photorespiration. Downton et al. (1990) reported a decrease in stomatal conductance and photosynthetic activity of salt-affected Sultana vines. On the other hand, Meggio et al. (2014) reported that Na^+ and Cl^- accumulation in grapevine tissues under salt stress caused a decrease in stomatal conductance and water potential. Some osmoprotectants, such as proline, observed in grapevines under salt stress are known to provide antioxidative properties, emphasizing the close relationship between water and oxidative stresses, especially under salinity, and the importance of a combined response accordingly (Ozden et al. 2009; Haider et al. 2019). Indeed, Fozouni et al. (2012) observed an increase in the concentration of compatible solutes such as soluble sugars and proline in grapevine leaves after saline irrigation.

5-ALA treatments provided significant increases in RWC ratio, stomatal conductance and proline content in both salinity stressed and non-salinity stressed groups compared to 5-ALA untreated groups. While all 5-ALA concentrations under salinity stress were found to be effective in increasing RWC ratio, 0.6 and 0.9 mM 5-ALA applications were found to be the most effective concentrations in terms of proline content and 0.9 mM 5-ALA application was found to be the most effective concentrations in terms of stomatal conductance. In the groups without salinity stress, 0.6 and 0.9 mM 5-ALA treatments were the most effective concentrations in terms of stomatal conductance; 0.9 mM 5-ALA treatment was the most effective in terms of RWC, proline content and leaf temperature. The reason why 5-ALA increased proline content, stomatal conductance and leaf relative water content in grapevine saplings was thought to be due to its ability to regulate osmotic balance in plant cells, water regulation and increase their ability to cope with stress (Tan et al., 2022). In parallel with our findings, Yang et al. (2014) reported that 5-ALA (0.5 $mg.l^{-1}$) sprayed on leaves against 200 mM NaCl stress in creeping bentgrass (*Agrostis stolonifera*), a salinity-sensitive perennial grass species, increased chlorophyll content, net photosynthetic rate, leaf relative water content and stomatal conductance, and was effective in alleviating membrane electrolyte leakage and lipid peroxidation damage caused by salinity stress. In a similar study, Youssef & Awad (2008) reported that 5-ALA increased the rate of photosynthesis by increasing leaf relative water content and stomatal conductance in date palm seedlings (*Phoenix dactylifera*) exposed to salinity stress. Tang et al. (2016) reported that foliar application of 5-ALA at concentrations of 0, 12.5, 16.7, 25.0 and 50.0 $mg.l^{-1}$ increased the fresh weight, chlorophyll content (SPAD), stomatal conductance and antioxidant enzyme activities of leaves and roots against salinity stress in *I. indigotica*. Manafi et al. (2015) determined that exogenous 5-ALA applications at 0.3 mM concentrations applied from seed and leaves increased stomatal conductance and relative water content in soybean (*Glycine max* L. Merr) against cold stress. On the other hand, Yang et al. (2021) reported that proline content and antioxidant enzyme activity increased in *Buxus megistopphylla* Levl exposed to various stress factors as a result of spraying leaves with 5-ALA at a concentration of 20 $mg.l^{-1}$.

Salinity stress caused significant increases in the physical damage, membrane damage and MDA content of grapevine saplings compared to non-stressed groups. This effect was thought to be due to increased oxidative stress associated with reactive oxygen species (ROS) (Fozouni et al. 2012).

5-ALA treatments provided a significant reduction in physical damage, membrane damage and MDA content under salinity stress compared to 5-ALA untreated groups. While all 5-ALA concentrations were found to be effective in reducing physical damage and MDA content under salinity stress, 0.6 and 0.9 mM 5-ALA treatments were found to be the most effective concentrations in reducing membrane damage. In the groups without salinity stress, 0.9 mM 5-ALA application was found to be the most effective concentration in terms of reducing membrane damage. In the present study, it was thought that the effect of 5-ALA applications on the reduction of salinity stress damage may be related to the increase in the amount of cell antioxidants and protection of plasma membranes against free radicals (Nishihara et al., 2003). Indeed, Wongkantrakorn et al. (2009) noted that a decrease in lipid peroxidation (MDA) was observed in NaCl-treated paddy (*Oryza sativa* L.) due to 5-ALA-induced activation of antioxidative enzymes.

In a similar study, Genişel & Erdal (2016) found that 5-ALA applications (10 and 20 $mg.l^{-1}$) significantly increased protein content and SOD, CAT and APX enzyme activities in wheat seedlings against 150 mM NaCl stress and significantly alleviated lipid peroxidation and stress-induced oxidative damage. On the other hand, Manafi et al. (2015) determined that 5-ALA applications at 0.6 mM concentrations applied from seed and leaf against cold stress in soybean (*Glycine max* L. Merr) increased SOD and CAT enzyme activities and proline amounts and reduced membrane damage. Hotta et al. (1998) and Zhang et al. (2006) reported that low 5-ALA concentrations increased cold tolerance in paddy and potato. In the present study, interestingly, all 5-ALA concentrations caused an increase in MDA content in the non-salinity stressed groups. In this increase in MDA content, it was thought that the excessive accumulation or unbalanced distribution of compatible solutes in the cell may have led to increased oxidative stress in cell membranes and triggered lipid peroxidation (Shen et al. 1999, Singh et al. 2015).

Conclusion

This study investigated the effects of exposure of 41 B American grapevine rootstocks to salinity stress and foliar application of different 5-ALA concentrations on morphological, physiological and biochemical traits. Salinity stress caused significant decreases in growth parameters, chlorophyll content and water balance, whereas 0.9 mM 5-ALA treatments resulted in significant increases in plant growth characteristics, chlorophyll content and water holding capacity. Moreover, the improvement in oxidative stress parameters emphasizes the potential of 5-ALA to increase salinity tolerance in plants. These results suggest that 5-ALA may be a promising alternative application for enhancing tolerance to salinity stress in agricultural fields. Among the different concentrations used in this study, 0.9 mM 5-ALA (high concentration) was found to give the best results. Therefore, it is recommended that higher concentrations be tested in future studies on the use of 5-ALA in grapevine.

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Quality Parameters and Antioxidant Activity, Phenolic Compounds, Sensory Properties of Functional Yogurt with Melon (*Cucumis melo* L.) Peel Powder

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ABSTRACT

In the current study, four different types of yogurt were produced as control samples (no MPP added) and 1, 2, and 3% melon peel powder (MPP1, MPP2, and MPP3). These yogurts were determined by physicochemical, microbiological, sensory, total phenolic, and antioxidant activity weekly for 21 days. While ash, moisture, titratable acidity (TA), viscosity, water holding capacity (WHC), *a** and *b** values, total phenolic content (TPC), and antioxidant capacity of melon peel powder samples increased, *L**, pH, and syneresis values decreased. In concentrations of 1, 2, and 3%, the mean antioxidant activity of powdered yogurt was found to have average values during storage of 30.09%, 32.32%, and 36.26%, respectively. All yogurts continued to contain more than 10^7 cfu/g of live lactic acid bacteria during fermentation. As the storage time increased, the sample's pH and syneresis decreased, while titration acidity and texture increased. No yeast or mold (2 log cfu/g) was determined in the samples. The panelists preferred MPP1 and MPP2 samples. According to the findings of the study, melon rind powder, which is a by-product, can be recommended as a functional food additive in yogurts.

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Introduction

The food business and the food service market are now attractive, high-income areas with significant investments. However, some businesses stand out with the amount of waste they create after production (Rolim et al., 2020). According to estimates, more than one-third of the world's food was wasted in 2022, and about 98 million tons of food were squandered in 2023 as of June 30. Furthermore, the direct economic repercussions of food waste amount to around $750 billion per year, excluding fish and shellfish (The World Counts, 2024; Rațu et al., 2023). Fruits and vegetables that have been processed yield waste materials like fiber and byproducts like peel and seeds (da Silva and Jorge, 2014; Mallek-Ayadi et al., 2017). Fruit peels contain many bioactive substances, and recovering them might be profitable. Goulas and Manganaris (2012), the peel of most fruits contain higher concentrations of phenolic compounds and ascorbic acid than the pulp does (Mallek-Ayadi and Kechaou, 2017). Thus, from many aspects, including functional new product creation, environmental protection, and economic development, the evaluation of by-products is both essential and significant (Comunian et al., 2021; Dinkçi et al., 2021). Because it adapts to varied soil and temperature types, the melon (*Cucumis melo* L.), a member of the Cucurbitaceae family, is a widely consumed fruit of economic significance farmed around the world (Rolim et al., 2018). With the advancements in the food industry, wastes from melon fruit, a by-product of the fruit industry, are employed in the manufacturing of numerous items such as fruit juices, prepared salads, and snacks (Lucas-Torres et al., 2016; Gómez-García et al., 2020). Hence, the processing of melon peel, which is the source of many valuable natural components like pectin, limonene, flavonoids, polymethoxy flavones, and carotenoids, is studied in melon processing companies (Li et al., 2006; Raji et al., 2017). According to studies, eating melon and its byproducts can help prevent and treat certain diseases, including cancer and inflammation. It can also be used as a natural remedy for aging (Shofian et al., 2011; Rodríguez-Pérez et al., 2013; Gómez-García et al., 2020). Moreover, consumers' awareness of healthy nutrition has led to the addition of melon peel powder, which is rich in bioactive compounds, to yogurt production.

Previous studies have reported the use of melon peel in different ways, but its incorporation into yogurt has not been studied. It is thought that the melon peel will provide functionality to yogurt with the components it contains. Additionally, MPP yogurt production could create an alternative for the food market. Therefore, the physicochemical, microbiological, and sensory properties of yogurts produced by adding different concentrations of melon peel powder were investigated.

Materials and Methods

Materials

As a direct-to-vat system yogurt culture, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus*, YC350 brand (Chr. Hansen-Peyma, Istanbul, Turkey) were employed. In the production, cow milk and fresh melon fruits (*Cucumis melo* L. var. *reticulatus*) from the region of Erzincan were used.

Preparation of Melon Peel Powder

Melon rinds (5 kg) were first washed, shredded, and then peeled with a stainless steel knife, and dehydrated in a domestic microwave oven (Arçelik KMF 833 l, Turkey) for 24 hours at 50 °C (Al-Sayed and Ahmed, 2013). A blender (Warning Commercial, USA) was used for grinding. To obtain uniform dimensions, these dried peels were ground in a food processor and put through 300 m sieves. Afterward, this powdered peel was concealed in hermetic bags at -18ºC.

Chemical and Physical Composition of Cow Milk and Melon Peel Powder

The dry matter content of milk used in yogurt production was determined gravimetrically at 105±2 °C, the pH value was determined by a digital pH meter, the acidity value was determined by the titration method with 0.1 N NaOH, and the ash determination was determined gravimetrically at 550 °C.

Melon peel powder dry matter was measured according to the Association of Official Analytical Chemists (AOAC) method. In the ash analysis, the samples were analyzed by burning in a muffle furnace at 550 for 4 hours. The pH values were determined with a digital pH meter (Eutech PH 150 Model) by diluting the melon fiber with distilled water at a ratio of 1:10 (m:v) (Grigelmo-Miguel and Martoan-Belloso, 1998). Color analysis was performed using a color measurement device (Chroma Meter, CR-5, Konica Minolta, Osaka, Japan; Dirim and Çalışkan, 2012).

Total phenolic content was determined using the Folin-Ciocalteu method (Singleton et al., 1999). For this purpose, 1000 µg of the extracts were taken, and the total volume was made up to 25 mL. The mixture was vortexed by adding 0.5 mL of Folin-Ciocalteu's reagent and 1.5 mL of 2% Na_2CO_3 at 3 minute intervals. The absorbance of the samples kept at room temperature and in the dark for 30 min was determined at 760 nm with a UV-vis spectrophotometer (Shimadzu, UV mini-1240, Japan), and the total phenolic content was expressed as mg GAE/g using the curve prepared with gallic acid (R2= 0.982).

In the study, 2.95 mg DPPH (2,2-diphenyl-1-picrylhydrazyl) was weighed and transferred to a 50 mL balloon jug and DPPH solution was prepared by filling the balloon jug with methanol (Merck, Germany) to the line. For analysis, 200 µL of the prepared extracts were taken and transferred to test tubes. After adding 3 mL of freshly prepared DPPH solution and vortexing for 30 seconds, it was kept for 30 minutes in a dark environment at room temperature. After this time, the absorbance was read at 517 nm on a UV-vis spectrophotometer (Shimadzu, UV mini-1240, Japan; Ye et al., 2013).

$$\text{DPPH (\%)} = \left(\frac{1 - \text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

Manufacture of Yogurt Samples

In the yogurt production, with the aid of Ultra Turrax (Daihan Scientific, Co., Ltd.), milk and melon peel powder were combined. Later, the milk was heated (90°C for 10 min.) and then cooled (42°C). The milk was split into four equal pieces. Three parts were produced with melon peel powder at various concentrations of 1%, 2%, and 3% (MPP1, MPP2 and MPP3), and a control (C). In sterile plastic containers, starting cultures were used to incubate yogurts at a temperature of 42ºC and analyzed during 21 days of storage. The images of trial yogurt samples are given in Figure 1.

Figure 1. The images of trial yogurt samples

Physical and Chemical Analysis

The method described by Kurt et al. (2007) was used to gravimetrically quantify the moisture, ash, and titration acidity values of melon peel powder. A pH meter (Eutech PH 150 Model) was used to determine the pH (AOAC, 1990). A viscometer with the Brookfield brand [model DV-1; Brookfield Engineering Laboratories, Inc., MA, USA); Gassem et al. 1991] was used to calculate the viscosity values. The serum separation and water-holding capacity were expressed using the techniques described by Delikanlı and Özcan (2014) and Remeuf et al. (2003) methods respectively.

Colour Measurement

The Hunter instrument (Colourflex-EZ, Hunterlab, Virginia, USA) was calibrated before the analysis began. The homogenized samples' *L**, *a**, and *b** color values were read (Cueva and Aryana, 2008). Using the following formulas, the saturation index (*C**), hue angle (*H°*), and total color difference (*ΔE**) were calculated (Kurtuldu and Özcan, 2018)

$$C^* = (a^{*2}+b^{*2})^{1/2}$$

$$H^0 = \tan^{-1}(b^*/a^*)$$

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

Determination of Total Antioxidant Activity

The 2,2-diphenyl-1-picrylhydrazyl method was modified to assess the samples' capacity to scavenge (DPPH) radicals (Ye et al., 2013).

DPPH (%)=(1-Absorbance of sample/Absorbance of control) x 100

Total Phenolic Content Assay (TPC)

Total phenolic levels were determined spectrophotometrically using the Folin-Ciocalteu technique (Singleton and Rossi, 1965). 150 µL of sample extracts were combined with 600 mL of 7.5% (w/v) Na_2CO_3 and 750 µL of Folin Ciocalteu reagent. To measure the absorbance, a UV/visible spectrophotometer was used at 765 nm (Perkin-Elmer, USA). The results are expressed as g of gallic acid equivalent (GAE) per gram of yogurt sample.

Microbiological Analysis

Using a Stomacher (Interscience-Bagmixer 400 P, St. Nom, Fransa), 10 g of yogurt samples were diluted in 90 mL of a 0.85% (w/v) NaCl solution. The Harrigan (1998) approach was used to find the yeast and mold cells. The agar plates were kept at room temperature for 5-7 days of incubation. M17 agar was used to test the *S. thermophilus* cells under aerobic conditions. It was incubated at 35-37 °C for 24-48 h. Using MRS agar, *L. bulgaricus* cells were counted after it was cultured anaerobically for 72 hours at 37 °C (Vinderola and Reinheimer, 1999).

Sensory Evaluation

Eight panelists participated in the sensory evaluation, rating the samples' appearance, color, flavor, texture, and general acceptability on a scale from 1 to 9 (poor to outstanding) throughout the storage period (Bodyfelt et al., 1988).

Statistical Analysis

The SPSS (Version 22.00, SPSS, IBM, NY, USA) package program was used to conduct a variance analysis of the results.

Results and Discussion

Physicochemical of Cow Milk and Melon Peel Powders

Table 1 presents the findings of the physicochemical analysis of cow milk and melon peel powdered. Dry matter (8.85±0.21%), total antioxidant activity (13.15±1.82%), and total phenolics (2.59±0.09 mg GAE/g) made up the melon peel powder's composition. In a study, the total phenolic content of mazoon melon peel was found to be 332 mg/100 g extract (Mallek-Ayadi et al., 2017). The melon peel, however, was likely higher in phenolic compounds than the seeds and flesh (285 and 168 mg/100g respectively), according to İsmail et al. (2010). According to Al-Sayed and Ahmed (2013), four phenolic compounds, including 4-hydroxybenzoic acid, chlorogenic acid, coumaric acid, and vanillin, were found in sharlyn melon peels. These compounds ranged in concentration from 66.2 to 325.3 µ/g DW. The researchers also determined that the melon peel's free radical scavenging activity (DPPH) value was 12.53%. In a study, pH (6.63±0.01a), titration acidity (0.23±0.02), dry matter (11.7±0.28), ash (0.45±0.02), fat (3.05±0.07b), and protein (3.11±0.03) of cow milk were found (Nalbant and Yüceer, 2020).

pH, Titratable Acidity, and Microbiological Counts

Table 2 lists the physicochemical characteristics of the samples. The treatments had a substantial impact on these characteristics ($p<0.05$). The lowest (1.038±0.004) and maximum (1.204±0.004) acidity values were found in the C and MPP3 samples, respectively. Melon peel powder raised the acidity values of yogurts. According to Wang et al. (2019), the added apple fiber did not significantly affect the titratable acidity values of set-type yogurts throughout the 28 days (up to only 0.15%). Perez-Chabela et al. (2021), yogurt samples made with mango peel flour exhibited higher titratable acidity than those from the control group. After 21 days of storage, they found that the acidity readings significantly rose ($p<0.05$). It was observed that pH decreased as the amount of melon peel powder in yogurt increased. It can be interpreted that melon peel powder supports microbial growth.

As pH decreased during fermentation, titratable acidity increased. Similarly, Kavak and Akdeniz (2019) stated that pH values in yogurt manufacture that had been supplemented with grape seed extract somewhat decreased. Moreover, during storage, pH values varied between samples with melon peel powder (1, 2, and 3%) and control yogurt. *Streptococcus thermophilus* and *Lactobacillus bulgaricus* are homofermentative bacteria that convert lactose to lactic acid. Lactic acid synthesis leads to a fall in pH, which is to be expected.

It is also feasible to enhance the qualities of yogurt, such as texture, by the formation of aromatic compounds or exopolysaccharides (EPSs) in the industrial sense thanks to the protocooperation interaction between *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Rul, 2017).

Table 1. The physicochemical analysis of cow milk and melon peel powdered

Analyses	Materials	
	Cow milk	Melon peel powder (MPP)
Total Solids (%)	12.40±0.13	8.85±0.21
Ash (%)	0.61±0.01	6.30±0.04
pH	6.60±0.00	5.19±0.02
Titratable acidity (%)	0.17±0.02	ND
Total Phenolics (mg GAE/g)	ND	2.59±0.09
DPPH	ND	13.15±1.82
*L**	ND	67.44±2.19
*a**	ND	2.61±0.16
*b**	ND	22.28±1.57

*ND Not detected; values are mean ± standard deviation

Table 2. Changes of pH, titratable acidity and count of bacteria in yogurt fortified with melon peel powder during storage at 4°C

Analyses	Applications	Storage Time (day)				Mean
		1	7	14	21	(X ± Sx)
pH	C	4.605	4.575	4.560	4.440	4.545 ± 0.009^{A}
	MPP1	4.545	4.345	4.195	4.140	4.306 ± 0.009^{B}
	MPP2	4.460	4.265	4.180	4.115	4.255 ± 0.009^{C}
	MPP3	4.405	4.220	4.120	4.040	4.196 ± 0.009^{D}
	Mean (X ±Sx)	4.504 ± 0.009^{a}	4.351 ± 0.009^{b}	4.264 ± 0.009^{c}	4.184 ± 0.009^{d}	
Titratable acidity	C	0.965	1.054	1.095	1.039	1.038 ± 0.004^{D}
	MPP1	1.095	1.125	1.147	1.195	1.140 ± 0.004^{C}
	MPP2	1.106	1.188	1.223	1.237	1.188 ± 0.004^{B}
	MPP3	1.140	1.193	1.228	1.257	1.204 ± 0.004^{A}
	Mean (X ±Sx)	1.077 ± 0.004^{c}	1.140 ± 0.004^{b}	1.173 ± 0.004^{a}	1.182 ± 0.004^{a}	
Lactobacillus bulgaric	C	8.800	8.405	8.000	7.830	8.259 ± 0.005^{D}
	MPP1	8.905	8.500	8.300	8.000	8.426 ± 0.005^{C}
	MPP2	8.940	8.600	8.400	8.200	8.535 ± 0.005^{A}
	MPP3	8.920	8.565	8.405	8.180	8.517 ± 0.005^{B}
	Mean (X ±Sx)	8.891 ± 0.005^{a}	8.518 ± 0.005^{b}	8.276 ± 0.005^{c}	8.052 ± 0.005^{d}	
Streptococcus thermophil	C	8.405	8.775	8.000	7.895	8.269 ± 0.10^{C}
	MPP1	8.440	8.800	8.040	8.000	8.320 ± 0.10^{B}
	MPP2	8.250	8.825	8.175	8.100	8.337 ± 0.10^{B}
	MPP3	8.650	8.805	8.150	8.065	8.417 ± 0.10^{A}
	Mean (X ±Sx)	8.436 ± 0.010^{b}	8.801 ± 0.010^{a}	8.091 ± 0.010^{c}	8.015 ± 0.010^{d}	

The horizontal column, lowercase letters (A-D), expresses differences between yogurt samples ($p<0.05$); the vertical column, capital letters (a-d), expresses differences between storage periods ($p<0.05$). C: Without melon peel powder; MPP1: 1% melon peel powder; MPP2: 2% melon peel powder; MPP3: 3% melon peel powder

Table 2 presents the findings from lactic acid bacteria and yeast-mold live cells during storage. *Streptococcus thermophilus* and *Lactobacillus bulgaricus* both had viable cell counts that ranged from 7.90 to 8.83 log cfu/mL and 7.83 to 8.94 log cfu/mL, respectively. These starter counts were observed to be lower in the control than in powdered samples. It was shown that during the storage period, there were fewer *L. bulgaricus* yogurts. *S. thermophilus* was found to be more prevalent overall, particularly in MPP3 of the powder-containing samples. Depending on the powder concentration, the number of viable cells of *L. bulgaricus* was assessed at most in 2% concentration and 3% concentration in *S. thermophilus* samples. When *L. bulgaricus* and *S. thermophilus* were tested in terms of storage, the least number of viable cells was found on day 21. Yogurt's enrichment with melon peel powder had a favorable impact on the development of the starting cultures. Perina et al. (2015) reported that after 14 days of preservation in probiotic yogurts with vegetable oil emulsion and powdered passion fruit peel, the average number of live *S. thermophilus* cells was 8.65±0.11 log cfu/g and *L. bulgaricus* as was 6.0 log cfu/g.

None of the yogurt samples during storage had any yeast or mold (<2 log cfu/g). The treatment successfully extends the shelf life of yogurt while also making it safer. The hygienic conditions used during processing and packing determine how long yogurt will last. According to Brahmi et al. (2021), yogurts containing apple peel had coliform, yeast, and mold levels of less than <10 log cfu/mL. These results show that the manufacturing was conducted in hygienic settings.

Dry matter, Ash, Viscosity, Syneresis and Water Holding Capacity (WHC)

Dry matter, ash, viscosity, syneresis and water holding capacity (WHC) are given in Table 3. Dry matter content was between 14.346%±0.015 and 15.381%±0.015. Particularly, it was found that the control sample (C) had less dry matter than the other samples. The ash was found at 0.925±0.03% and 1.204±0.03%. As expected, increasing the melon peel powder content led to an increase in the dry matter and ash values.

Melon peel powder was shown to significantly affect syneresis ($p<0.05$), with values ranging from 4.912±0.0128 to 8.019±0.0128 (Table 3). The control yielded the highest syneresis value, whereas the MPP3 sample yielded the lowest syneresis. Kabir et al. (2021), found no discernible difference between the yogurt control and banana peel extract samples ($p>0.05$). Garcia-Perez et al. (2005), the percentage of orange fiber had a significant impact on the yogurts' syneresis values ($p<0.05$). Adding 0.6% and 0.8% fiber caused the gel structure to break down, which increased the syneresis values. The high WHC of the fiber, which absorbs the water exiting the gel structure when 1% fiber is introduced, was responsible for this impact, though. On the other hand, it was found that during cold storage, syneresis values rose in all yogurt variants ($p<0.05$). As the storage duration increased, the amount of syneresis decreased.

Unlike syneresis, WHC is a very important physical measurement, as only details of the hardness and stability of coagulants reflect consumer preferences. The yogurt samples' water retaining capacities were measured; the control had the lowest value (50.069±0.229), and the MPP3 had the highest value (58.186±0.229). Moreover, the WHC rose with the rate of MPP addition. It was hypothesized that this would be because the powdered melon peel has a high water absorption capacity and binds more water while in storage. The WHC of yogurt samples was found by Ahmad et al. (2020) to be 53.67% in control, 56.10% in yogurt with 1% apple peel extract, and 66.23% in yogurt with 5% apple peel extract. Yogurts made from camel milk utilizing banana and peel fiber showed WHC, according to Safdari et al. (2021). They mentioned that the high concentration of water-soluble fiber might be to blame for this outcome.

Viscosity is a crucial metric that reveals details about the yogurt's consistency, clot stability, and quality. The control sample had the lowest viscosity value of the samples (2578.00±327.71 cP), while the MPP3 sample had the highest value (2578.00±327.71 cP). The rise in viscosity in samples containing melon peel powder may be the result of the powder absorbing water. Ahmad et al. (2020), the inclusion of apple peel increased the samples' hardness and viscosity while reducing their syneresis. Demirkol and Tarakçı (2018) research, grape pomace powder-enriched yogurts had lower viscosity values than control yogurts. According to Tseng and Zhao (2013), dietary fiber supplied from wine grapes enhanced the viscosity parameters of yogurts. Researchers hypothesized that this rise may be caused by increased milk coagulation during yogurt manufacturing. Nevertheless, Manzoor et al. (2019) produced yogurt with concentrations of 1.5% and 3.0% w/w after drying papaya peel powder at two different temperature ranges, 55 ºC (PP1) and 65 ºC (PP2). According to their findings, samples made with 3% powder (3.0% PP1 and 3.0% PP2) had higher viscosities than samples made with 1.5% powder (1.5% PP1 and 1.5% PP2). Consistency was found between the researchers' findings and the data from this study. When the samples were analyzed in terms of storage, dry matter and ash increased while syneresis and WHC decreased as the storage period increased.

Total Phenolics Content (TPC)

The TPC values of yogurt powdered ranged from 2.50±0.00 to 4.80±0.01 g GAE/100 g (Fig. 1). All of the powdered yogurts showed statistically significant differences ($p<0.05$). By day 21, MPP3 (4.80±0.01 g GAE/100 g) had the highest phenolic content, followed by MPP2 (4.52±0.00 g GAE/100 g), MPP1 (3.96±0.01 g GAE/100 g), control (3.00±0.01 g GAE/100 g). The results showed that the total phenolic content of yogurts increases depending on the amount of melon peel powder concentration.

These outcomes unequivocally demonstrated that the enhanced yogurt polyphenols from melon peel powder might be used as bioactive ingredients in food preparation. According to Ahmad et al. (2020), the control yogurt had a total phenolic content of 1.48 g GAE/100 g DW on the first day of storage. The researchers claimed that the TPC of yogurt samples (3.54, 4.76, 6.11, 7.45, and 8.94 g GAE/100g of DW, respectively) rose with the content of apple peel polyphenol extract (1%, 2%, 3%, 4%, and 5%). In addition, according to the researchers, TPC content rose during the first two weeks while falling during the ensuing second and third weeks. They mentioned that the statistical correlation of the total antioxidant activity of apple peel might be the cause of these results. The phenolic content of yogurts containing passion fruit ranged from 0.50 mg/100 g GAE to 8.01 mg/100 g GAE according to Asiimwe et al. (2021). Kabir et al. (2021), the concentration increase caused the TPC of yogurts containing banana peel extract to rise. It was evaluated that yogurt samples were enhanced with green coffee and green tea powder and had their total phenolic content over time (Dönmez et al., 2017). In addition, the current study results concur with those of the researchers mentioned above.

Table 3. Physicochemical characteristics of yogurt fortified with melon peel powder

Analyses	Applications	Storage Time (day)				Mean (X ± Sx)
		1	7	14	21	
Dry matter (%)	C	14.055	14.305	14.455	14.570	$14.346±0.015^{D}$
	MPP1	14.670	14.915	15.145	15.205	$14.984±0.015^{C}$
	MPP2	14.950	15.115	15.215	15.355	$15.159±0.015^{B}$
	MPP3	15.270	15.300	15.425	15.530	$15.381±0.015^{A}$
	Mean	$14.736±0.015^{d}$	$14.909±0.015^{c}$	$15.060±0.015^{b}$	$15.165±0.015^{a}$	(X ±Sx)
Ash (%)	C	0.890	0.905	0.930	0.975	$0.925±0.003^{D}$
	MPP1	0.965	1.000	1.100	1.120	$1.046±0.003^{C}$
	MPP2	1.085	1.130	1.155	1.200	$1.143±0.003^{B}$
	MPP3	1.160	1.200	1.240	1.215	$1.204±0.003^{A}$
	Mean	$1.025±0.003^{d}$	$1.059±0.003^{c}$	$1.106±0.003^{b}$	$1.127±0.003^{a}$	(X ±Sx)
Viscocity (cP)	C	2199.50	2399.50	2772.50	2940.50	$2578.00±327.71^{B}$
	MPP1	3017.50	3630.50	3834.00	3865.00	$3586.75±327.71^{AB}$
	MPP2	1743.50	3710.50	3953.00	2025.50	$2858.13±327.71^{B}$
	MPP3	3589.00	3937.00	4147.50	4332.00	$4001.38±327.71^{A}$
	Mean	$2637.38±327.71^{a}$	$3419.38±327.71^{a}$	$3676.75±327.71^{a}$	$3290.75±327.71^{a}$	(X ±Sx)
Syneresis (%)	C	9.330	8.975	6.975	6.795	$8.019±0.128^{A}$
	MPP1	8.850	7.325	7.850	6.225	$7.562±0.128^{B}$
	MPP2	6.855	6.100	5.700	4.750	$5851±0.128^{C}$
	MPP3	6.050	5.850	4.100	3.650	$4.912±0.128^{D}$
	Mean	$7.771±0.128^{a}$	$7.063±0.128^{b}$	$6.156±0.128^{c}$	$5.355±0.128^{d}$	(X ±Sx)
WHC (%)	C	53.805	51.125	48.945	46.400	$50.069±0.229^{D}$
	MPP1	56.240	55.160	53.090	51.350	$53.960±0.229^{C}$
	MPP2	59.035	57.005	54.125	51.610	$55.444±0.229^{B}$
	MPP3	62.210	59.590	57.855	53.090	$58.186±0.229^{A}$
	Mean	$57.823±0.229^{a}$	$55.720±0.229^{b}$	$53.504±0.229^{c}$	$50.613±0.229^{d}$	(X ±Sx)

The horizontal column, lowercase letters (A-D), expresses differences between yogurt samples ($p<0.05$); the vertical column, capital letters (a-d), expresses differences between storage periods ($p<0.05$). C: Without melon peel powder; MPP1: 1% melon peel powder; MPP2: 2% melon peel powder; MPP3: 3% melon peel powder

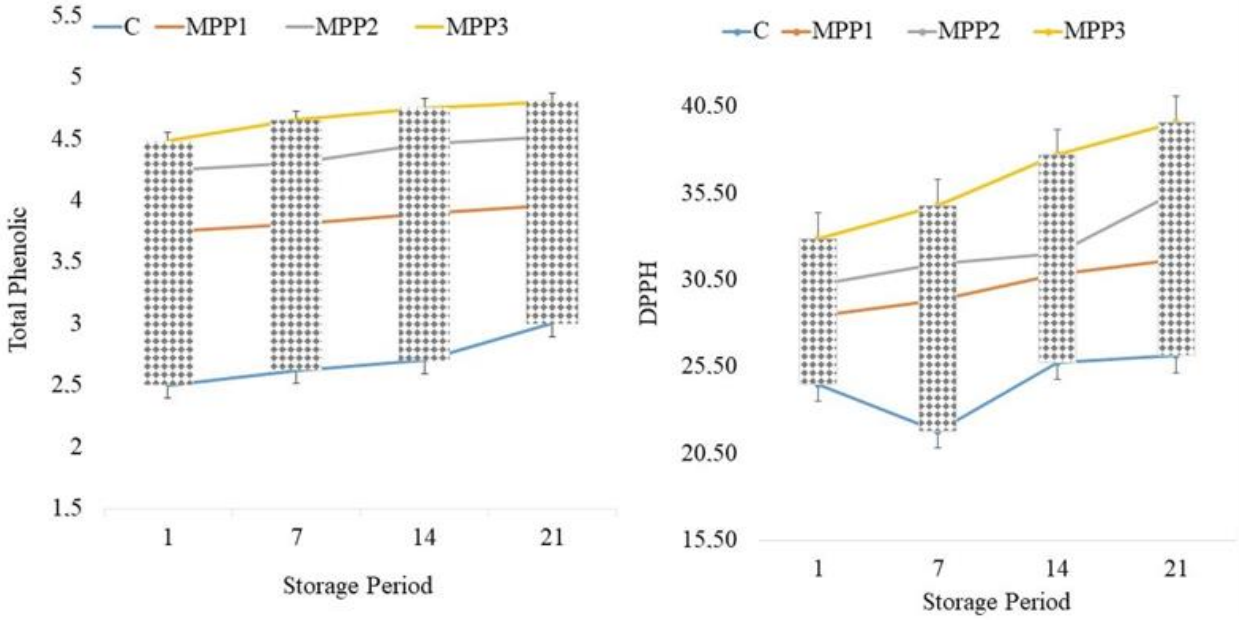

Figure 2. Total phenolic content (g GAE/100g DW) and DPPH (%) contents of yogurt enriched melon peel powder

Antioxidant Activity

In terms of their antiradical capacity (DPPH), yogurts were found to differ significantly ($p<0.05$). While control yogurt had the lowest activity, MPP3 yogurt had the best antioxidant potential. The average antioxidant activity values of yogurt with melon peel powder at 1%, 2% and 3% concentrations were 28.46%, 30.23% and 32.9%, respectively, on the first day of storage, while at the end of storage they were found to be 31.70%, 35.57%, and 39.59% (Fig. 1). Yogurt's higher MPP content had a beneficial impact on its antioxidant capacity. It might highlight yogurts containing melon peel powder, which has significant antioxidant activity due to its high phenolic content and could be employed as a new component in the design of nutraceuticals as well as a natural source of antioxidants. Super red dragon fruit skin was used to make yogurt by Supriyanti and Zackiyah (2020) (F1, F2, and F3 samples were created with 10, 20, and 30% concentrations, respectively). They identified the samples' antioxidant activity as F3, F2, F1, and F0, in order of highest to lowest. Also, they found that the antioxidant activity of the control sample was 15.68% and that it ranged between 63.69% and 92.67% in the samples made with enhanced powder. They found that the antioxidant activity in yogurts with a passion fruit flavor was much higher ($p<0.05$) and increased ($p<0.05$) after storage (Asimwe et al., 2021). Kabir et al. (2021), the condition causes an increase in the radical scavenging ability in both $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ values due to the increase in the concentration of banana peel extract. In comparison to control yogurts (27.13±1.53%), probiotic yogurts containing apple peel polyphenol extract had greater antioxidant activity (Ahmad et al., 2020). This study's results agreed with those of other studies.

Colour Values (L*, a*, b*, ΔE*, H° ve C*)

The L^*, a^*, and b^* values of samples were significantly impacted by enrichment ($p<0.05$), and it was determined that the samples with powder additions had lower L^* values than the control (Table 4). The whiteness of yogurts decreased because of the powder addition, as was to be predicted. In addition, it was shown that the drop in L^* values correlated with an increase in powder ratio. On the 21st day of storage, MPP3 had the lowest L^* value (66.85), whereas on the first day, powder-free C had the greatest L^* value (88.78).

The highest a^* value was observed in MPP3 (4.565) and the lowest in the control group (1.655). At the $p<0.05$ level, it was determined that the addition of melon peel powder and the length of storage had a substantial impact on the a^* value. The first day in MPP3 had the highest b^* value (22.64), whereas the first day in C had the lowest b^* value (12.30). The addition rates of melon peel powder were found to affect b^* values, and significant differences were found between the samples that contained the powder and the control group ($p<0.05$). The color study of melon peel powder yielded a b^* value of 22.28 (Table 1).

Thus, it is assumed that the samples' high b^* values are caused by the melon peel's natural yellowish hue. By increasing the percentage of orange fiber in yogurts, García-Pérez et al. (2005) found a rise in a^* (fewer greens) and b^* values (more yellowness) and a drop in L^* values (less whiteness). L^* values dropped with an increase in the concentration of banana peel extracts (Kabir et al., 2021). Furthermore, as the concentration of banana peel extract in yogurts increased, a* values rose (from 2.64 to 2.72) and b* values fell (from 22.65 to 19.81). During storage, it was detected that the L^*, a^* and b^* values of enriched yogurts became somewhat darker ($\Delta L^*<0$), redder ($\Delta a^*>0$), and less yellow ($\Delta b^*<0$). Adding grape seed extract to yogurt during storage results in a dark brown or reddish color ($\Delta L^*<0$, $\Delta a^*>0$, and $\Delta b^*<0$) (Yadav et al., 2018). On the other hand, yogurt made with orange peel fiber had an L^* value of 89 (Mary et al., 2022). In addition, they evaluated that the L^* values of samples of yogurt containing 0.25% (w/v) of concentrate improved more than controls, while the L^* values of samples of yogurt containing 0.1% (w/v) of partly hydrolyzed guar gum (PHGG) declined. Both the control and fiber-added samples' color change values (ΔE^*) were altered while being stored.

It was found that yogurt sample differences in color difference (ΔE^*), hue value (H^o), and saturation index (C^*) values were statistically significant ($p<0.05$). It was

determined that there were variations in the control sample and a general decline in the *C** values of the yogurt samples throughout storage. The *C** value increased along with the melon peel powder content. According to Manzoor et al. (2019), the *C** value of yogurts with papaya peel powder added increased with concentration, while the *C** value of the control was significantly lower than the samples with 1% and 3% powder added. The lowest H^o value (77.44) was found in the MPP3 sample on the first day, and the greatest H^o value (83.68) was found in the control sample on the 21st day. In their investigation of the impacts of pineapple peel powder on yogurt, Shah et al. (2016) found that the H^o value of the yogurts was lower than that of the control. The color results in this study were evaluated following the literature.

Sensory Evaluation

All the sensory qualities considered were impacted by the addition of melon peel powder to yogurt at various rates ($p<0.01$). Storage times had a statistically significant impact on sample appearance scores ($p<0.05$). The MPP3 sample received the lowest scores from the panelists for look and color, 7.45 and 6.83, respectively (Table 2). The analysis of variance revealed that the impact of powder addition on yogurt scores for appearance and color was significant at the $p<0.05$ level. Chouchouli et al. (2013) found that the enrichment of yogurt with grape seed powder created a darker color than the control sample.

The MPP3 sample received the lowest flavor rating (7.40), while the C sample received the highest rating (7.88). The fact that the panelists tasted melon, even in a modest amount, positively affected their flavor ratings. The panelists preferred yogurts with 1% and 2% melon peel powder in particular. Moreover, the rise in acidity with time in storage may be to blame for the decline in flavor ratings. According to Tseng and Zhao (2013), yogurts with 1% wine grape pomace powder received higher ratings for taste and consistency.

Each member of the panel gave samples that contained 1% melon peel powder with greater texture scores. The increase in texture scores can be explained by the lower levels of syneresis in these samples. The yogurt with 2% melon peel powder (MPP2) received the best overall acceptance score (6.86), while the yogurt with no powder received the lowest overall acceptability score (6.50). A broad meaning of the phrase "general acceptability" encompasses sensory judgments like flavor, scent, consistency, and texture. In this regard, yogurt containing 2% powder in the sensory acceptance test (Table 2) had higher acceptance scores than other samples, followed by MPP1, MPP3, and C, respectively.

Table 4. Changes in color parameters in yogurt fortified with melon peel powder during storage

Parameters	Applications	Storage Time (day)				Mean (X ± Sx)
		1	7	14	21	
*L**	C	88.785	88.645	88.295	87.905	88.408±0.003^{A}
	MPP1	70.275	70.140	70.040	69.895	70.088±0.003^{B}
	MPP2	68.715	68.545	67.805	67.495	68.140±0.003^{C}
	MPP3	67.405	67.540	67.300	66.850	67.274±0.003^{D}
	Mean	73.795±0.003^{a}	73.718±0.003^{b}	73.360±0.003^{c}	73.036±0.003^{d}	(X ±Sx)
*a**	C	1.925	1.775	1.535	1.385	1.655±0.005^{D}
	MPP1	2.735	3.030	2.555	2.495	2.704±0.005^{C}
	MPP2	3.945	3.825	3.605	3.285	3.665±0.005^{B}
	MPP3	5.045	4.645	4.405	4.165	4.565±0.005^{A}
	Mean	3.413±0.005^{a}	3.319±0.005^{b}	3.025±0.005^{c}	2.833±0.005^{d}	(X ±Sx)
*b**	C	12.405	12.745	12.305	12.500	12.489±0.004^{D}
	MPP1	18.545	18.385	18.260	18.015	18.301±0.004^{C}
	MPP2	20.645	20.475	19.740	19.500	20.090±0.004^{B}
	MPP3	22.640	22.505	22.445	22.130	22.430±0.004^{A}
	Mean	18.559±0.004^{a}	18.528±0.004^{b}	18.188±0.004^{c}	18.036±0.004^{d}	(X ±Sx)
*C**	C	12.545	12.885	12.400	12.565	12.599±0.002^{D}
	MPP1	18.735	18.615	18.445	18.205	18.500±0.002^{C}
	MPP2	21.015	20.825	20.055	19.765	20.415±0.002^{B}
	MPP3	23.200	22.985	22.855	22.535	22.894±0.002^{A}
	Mean	18.874±0.002^{a}	18.828±0.002^{b}	18.439±0.002^{c}	18.267±0.002^{d}	(X ±Sx)
H^0	C	81.103	82.071	82.889	83.677	82.435±0.02^{A}
	MPP1	81.610	80.641	82.034	82.115	81.600±0.02^{B}
	MPP2	79.182	79.418	79.650	80.437	79.672±0.02^{C}
	MPP3	77.438	78.338	78.896	79.341	78.503±0.02^{D}
	Mean	79.833±0.020^{d}	80.117±0.020^{c}	80.867±0.020^{b}	81.392±0.20^{a}	(X ±Sx)
Δ*E**	C	89.685	89.585	89.155	88.805	89.308±0.003^{A}
	MPP1	72.735	72.555	72.410	72.210	72.478±0.003^{B}
	MPP2	71.865	71.645	70.705	70.315	71.133±0.003^{C}
	MPP3	71.285	71.325	71.075	70.545	71.058±0.003^{D}
	Mean	76.393±0.003^{a}	76.278±0.003^{b}	75.836±0.003^{c}	75.469±0.003^{d}	(X ±Sx)

The horizontal column, lowercase letters (A-D), expresses differences between yogurt samples ($p<0.05$); the vertical column, capital letters (a-d), expresses differences between storage periods ($p<0.05$). C: Without melon peel powder; MPP1: 1% melon peel powder; MPP2: 2% melon peel powder; MPP3: 3% melon peel powder

Table 5. Sensory properties of yogurt fortified with melon peel powder during storage

Parameters	Applications	Storage Time (day)				Mean (X ± Sx)
		1	7	14	21	
Appearance	C	8.455	8.305	8.060	8.250	8.268±0.003^{A}
	MPP1	8.000	7.810	7.660	8.000	7.868±0.003^{B}
	MPP2	7.950	8.050	7.800	7.370	7.793±0.003^{C}
	MPP3	8.000	7.860	7.400	6.540	7.450±0.003^{D}
	Mean	8.101±0.003^{a}	8.006±0.003^{b}	7.730±0.003^{c}	7.540±0.003^{d}	(X ±Sx)
Colour	C	6.300	7.325	7.690	8.000	7.329±0.005^{A}
	MPP1	6.165	7.100	7.550	7.830	7.161±0.005^{B}
	MPP2	6.065	7.000	7.405	7.555	7.006±0.005^{C}
	MPP3	6.000	6.840	7.200	7.310	6.837±0.005^{D}
	Mean	6.132±0.005^{d}	7.066±0.005^{c}	7.461±0.005^{b}	7.674±0.005^{a}	(X ±Sx)
Flavour	C	7.500	7.900	8.000	8.100	7.875±0.009^{A}
	MPP1	7.150	7.725	7.825	8.005	7.676±0.009^{B}
	MPP2	7.000	7.590	7.675	7.900	7.541±0.009^{C}
	MPP3	6.910	7.410	7.500	7.780	7.400±0.009^{D}
	Mean	7.140±0.009^{d}	7.656±0.009^{c}	7.750±0.009^{b}	7.946±0.009^{a}	(X ±Sx)
Texture	C	6.800	7.900	8.000	8.150	7.713±0.01^{A}
	MPP1	6.500	7.690	7.940	8.030	7.540±0.01^{B}
	MPP2	6.300	7.400	7.590	7.810	7.275±0.01^{C}
	MPP3	5.850	7.205	7.300	7.430	6.946±0.01^{D}
	Mean	6.362±0.010^{d}	7.549±0.010^{c}	7.707±0.010^{b}	7.855±0.010^{a}	(X ±Sx)
Acceptability	C	6.805	6.635	6.440	6.100	6.495±0.005^{D}
	MPP1	6865	6.805	6.740	6.300	6.678±0.005^{B}
	MPP2	7.000	6.950	7.000	6.475	6.856±0.005^{A}
	MPP3	6.905	6.705	6.600	6.065	6.569±0.005^{C}
	Mean (X ±Sx)	6.894±0.005^{a}	6.774±0.005^{b}	6.695±0.005^{c}	6.235±0.005^{d}	

The horizontal column, lowercase letters (A-D), expresses differences between yogurt samples ($p<0.05$); the vertical column, capital letters (a-d), expresses differences between storage periods ($p<0.05$). C: Without melon peel powder; MPP1: 1% melon peel powder; MPP2: 2% melon peel powder; MPP3: 3% melon peel powder

Yogurts supplemented with grape seed powder were better liked than the control, and those made with apple peel powder were less well-liked, according to the Brahmi et al. (2021) report. This outcome might be due to the smoother texture of the yogurts made with grape seed powder compared to the control yogurts, which also contained apple peel powder. The sensory criteria (taste, color, texture, consistency, flavor, and general acceptability) of yogurts with dried passion fruit powder have not been shown to change statistically significantly ($p>0.05$) during storage (Asiimwe et al., 2021).

Conclusion

The effects on the quality and storage durability of yogurt samples were examined in this study using various amounts of yogurt made with melon peel powder. It was shown that the dry matter and ash values statistically increased with the addition of powder in tandem with the increase in concentration ($p<0.05$). MPP-enriched yogurt substantially differed from all other samples' a^* and b^* values during storage ($p<0.05$). When melon peel powder (1, 2, or 3%) was added to yogurt, the pH, titratable acidity, and viable cell counts changed. Moreover, during storage, syneresis values declined while WHC and TPC values rose. With an increase in powder concentration, antioxidant activity and total phenolic contents rose. Yet, compared to the control yogurt sample, all of the powder-enriched yogurts had increased antioxidant activity.

Among the samples, the yogurt with 2% melon peel powder added had the greatest taste profile and highest sensory ratings. The study's findings suggested that adding melon peel powder to product preparation could enhance yogurt's beneficial qualities and help minimize food waste to safeguard the environment.

Declarations

Conflict of Interest The authors declare that they have no competing conficts of interest.

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Assessing Impact of Manual Topping and Suckericide Application at Different Stages on FCV Tobacco Quality and Yield

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ABSTRACT

Tobacco (*Nicotiana tabacum* L.) topping is one of the essential practice to obtain good quality and adequate yield. The suckericide (Flumetralin as a best herbicide and plant growth regulator). For the purpose to compare topping as manual and herbicide application the experiment was conducted on flue cured Virginia (FCV) tobacco at Tobacco Research Station, Khan Ghari, Mardan during 2021-2022. The experiment was conducted in RCB design with three replications and five treatments (Control, Manual de-suckering, 1000, 1200, 1500 ml of Flumetralin ha^{-1}). Treatments were applied at three stages (button stage, early flowering stage and late flowering stage). The data revealed that topping timing and maximum dose (1500 ml ha^{-1}) of (Flumetralin) resulted maximum (896 cm^{-2}) leaf area, less number (14) of sucker $plant^{-1}$, lower (132 g) green weight and dry weight (20.30 g) of sucker $plot^{-1}$, more cured weight (5.08 kg) of leaves $plot^{-1}$ and maximum yield (3038) kg ha^{-1}, lower nicotine contents (2.26) and less sugar contents (16.24) at button stage. Moreover, Flumetralin application on at button stage resulted less sucker growth and enhanced leaf yield. I suggest that the tobacco K399 with the application of suckericide and growth regulator (Flumetralin) have the potential to incorporate in further breeding program for low content of nicotine, reduced sugar content and high yield.

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Introduction

Tobacco (*Nicotiana tabacum* L.) is one of the most important cash crop of Pakistan, which contributes to the development of economy. Tobacco was grown in Pakistan, on about 50,800 hectares with production of about 113.6 million kilograms (PTB 2021). Tobacco is mainly cultivated for obtaining leaf because the leaf is manufacturing food material which is utilized in growth processes.

Topping (removal of the flowering head and young leaves) is an essential cultivation measure for tobacco growers, which switches off the plant from reproductive to vegetative phase (Gooden et al., 2011). Topping increases the size and weight of leaves, increasing the overall yield per hectare (Reed et al., 2012).

To encourage and maximize the leaf production and leaf ripening it is compulsory operation to remove the flower from the plant. The benefits of removing flower, switches off the plant from the seed production/reproductive phase to vegetative stage and it also enhancing leaf production/ vegetative phase (Pandeya et al., 2001; Wange et al., 2012).

Topping increases the size and weight of leaves, increasing the overall yield per hectare (Roton et al., 2005). The main aim of topping is to convert all the essential nutrients of the plant to the leaves instead of flowers and seeds which resulted of receiving in the large and better size of the tobacco leaf. It also provides proper and uniform quality of product and prevents extreme coarseness in the leaves (Wang et al., 2012).

In tobacco there are three important stages of topping operation i.e. button stage, early stage and late stage. Button stage is very imperative and economical stage of topping because it reduces weight from the top of the plant, increases root growth by reducing the movement of moisture and nutrient from lower leaves to support upper leaves, increase the nicotine contents and leaf size and therefore promote overall yield per hectare and especially for improve the plant growth, development and quality (Sadri and Zade et al., 2014).

For getting good quality yield from tobacco crop de-suckering is a compulsory process. Removing these suckers by hand is very laborious and time consuming job (Bakht et al., 2007). While chemical topping appears to be the ideal for removing excess leaves and the top which become flower in future. Therefore, in the current study we will investigate the influence of suckericide (Flumetralin) and manual topping on tobacco crop at different stages.

Material and Methods

A field experiment was conducted to check the influence of Flumetralin suckericide having different concentration at different growth stages of FCV tobacco K399 to evaluate the yield and quality. The research was laid out in RCB design having five treatments and three replications at Tobacco Research Center Khan Garhi Mardan during 2021-22, having a humid subtropical, no dry season climate average temperature is 18.06ºC (64.51ºF) and it is -2.83% lower than Pakistan's averages typically receives about 121.24 millimeters (4.77 inches) of precipitation and has 133.98 rainy days (36.71% of the time) annually. Soil is mostly silty loam with 8.0 to 8.3 pH and 0.10 to 0.17 (DSM^{-1}) electrical conductivity. Total soluble salts are in the range of 0.032 to 0.054%. CaCO3 is with a range of 6.50 to 9.0.and 0.62 to 0.89 % with organic matter content. Plants were sown in 90 cm row to row distance and 60 cm plant to plant distances. Plot size was 4×5 m^2 having 3 rows, and each row was 6 m long which was consists ten plants. The total research plot size was 300 m^2. In December, 2021 the nursery was raised for transplantation and transplanted during the first week of March, 2022. Data were recorded on leaf area (cm^2), number of suckers' $plant^{-1,}$ green weight (kg) of suckers' $plot^{-1,}$ dry weight of sucker $plot^{-1}$, cured weight of leaves plot (kg), Yield (kg ha^{-1}), Nicotine (%) and Reducing sugar (%)

The following treatment combination were used:

T_0 = Control

T_1 = Manual de-suckering

T_2 = 1000 ml ha^{-1} (Minimum dose)

T_3 = 1200 ml ha^{-1} (Optimum dose)

T_4 = 1500 ml ha^{-1} (Maximum dose)

These all five treatments were applied three times at button stage, early stage and late stage.

Statistical Analysis

The data were analyzed by using ANOVA and the methods described by (Jan et al., 2009) and for means comparison between the treatments least significant difference ($P \leq 0.05$) test were used.

Result and Discussion

Leaf Area (cm^2)

Different time of topping, and suckericide concentration extensively influenced the leaf area of tobacco crop. Maximum leaf area (1015.3 cm^2) with highest dose, (968 cm^2) with moderate dose followed by minimum dose and manual de-suckering, at button stage of topping (Table 1). The lowest leaf area (701.1 cm^2) was recorded from plots that were manually de-suckered at late flowering stage. The Interaction between maximum dose of Flumetralin and button stage topping indicated maximum leaf area in comparison with those plots which were manually de-suckered. It means that increasing suckericide concentration lead to increased leaf area of tobacco plant. Former findings of (Singh et al., 2000) reported that timely topping is an important operation for suckers control and to obtain better leaf area in tobacco crop. The earlier results of (Mahadevareddy et al.,1986) also noted that utilization of chemical suckericide have the best sucker control and leaf yield.

Number of Suckers' $plant^{-1}$

Number of suckers' $plant^{-1}$ were significantly affected by topping time and Flumetralin concentration. Plants where topping was done at button stage produced lowest sucker $plant^{-1}$ by maximum dose of Flumetralin (3.6) followed by optimum dose at button stage (8). Topping at later flowering stage have resulted highest sucker $plant^{-1}$ (32.6) by manual de-suckering. The interaction between the Flumetralin concentration and topping stages were significant as shown in (Table 2). Higher Flumetralin concentration application at button stage has resulted in better cover of plant and reduced new suckers' growth compared with late stage. The researcher (Farrokh et al., 2012) noted that topping plays a pivotal role in triggering the production of secondary plant compounds that gather in the foliage, making it imperative to perform this operation promptly in order to manage the growth of suckers effectively.

Green Weight (kg) of Suckers' $plot^{-1}$

Different time of topping and suckericide concentration significantly affected fresh weight of suckers. Interaction of maximum dose of Flumetralin at button stage of topping were also founded significant.

Table 1. Leaf area (cm^2) as effected by manual topping and suckericide application at different stage on FCV tobacco quality and yield.

Treatments	Button stage	Early stage	Late stage	Mean
T0 (control)	937	852.3	747.3	845.6 cd
T1 (Manual de-suckering)	941.7	826.7	701.1	823.3 d
T2 (Minimum dose)	951.3	859.3	765	858.6 bc
T3 (Optimum dose)	968.3	865.3	781.3	871.7 b
T4 (Maximum dose)	1015.3	884.3	788.3	896 a
Mean	962.7 a	857.6 b	756.7 c	

Mean values in the same category, marked by different letters, demonstrate a statistically significant distinction from one another, as evidenced by the LSD test, with a significance level of P<0.05.; Least significant difference value for treatments at 0.05 level = 17.34; Least significant difference value for treatments at 0.05 level = 13.43

Table 2. Number of suckers per plant^{-1} as affected by manual topping and suckericide application at different stage on FCV tobacco quality and yield

Treatments	Button stage	Early stage	Late stage	Mean
T0 (control)	28.6	33.3	34.3	32.1 a
T1 (Manual de-suckering)	27	31.6	32.6	30.4 a
T2 (Minimum dose)	19.6	26.3	21.3	22.4 b
T3 (Optimum dose)	8	22	23.6	17.8 c
T4 (Maximum dose)	3.6	19	20.6	14.4 d
Mean	17.4 b	26.4 a	27 a	

Mean values in the same category, marked by different letters, demonstrate statistically significant distinction from one another, as evidenced by the LSD test, with a significance level of P<0.05.; Least significant difference value for treatments at 0.05 level = 1.86; Least significant difference value for treatments at 0.05 level = 1.44

Table. 3 Green weight of suckers per plot^{-1} as affected by manual topping and suckericide Application at different stage on FCV tobacco quality and yield.

Treatments	Button stage	Early stage	Late stage	Mean
T0 (control)	215	247	255	239 a
T1 (Manual de-suckering)	180	234	238	217 b
T2 (Minimum dose)	144	188	191	174 c
T3 (Optimum dose)	99.3	170	175	148 d
T4 (Maximum dose)	72.7	160	163	132 e
Mean	142 b	200 a	204 a	

Mean values in the same category, marked by different letters, demonstrate a statistically significant distinction from one another, as evidenced by the LSD test, with a significance level of P<0.05; Least significant difference value for treatments at 0.05 level = 11.66; Least significant difference value for treatments at 0.05 level = 9.03

Table. 4 Dry weight of sucker per plot^{-1} as affected by manual topping and suckericide Application at different stage on FCV tobacco quality and yield.

Treatments	Button stage	Early stage	Late stage	Mean
T0 (control)	34.93	38.04	39.22	37.40 a
T1 (Manual de-suckering)	29.99	35.99	36.56	34.18 b
T2 (Minimum dose)	22.09	28.92	29.43	26.81 c
T3 (Optimum dose)	15.27	26.16	26.92	22.78 d
T4 (Maximum dose)	11.17	24.61	25.12	20.30 e
Mean	22.69 b	30.74 a	31.45 a	

Mean values in the same category, marked by different letters, demonstrate a statistically significant distinction from one another, as evidenced by the LSD test, with a significance level of P<0.05; Least significant difference value for treatments at 0.05 level = 1.87; Least significant difference value for treatments at 0.05 level = 1.45

Plots treated with maximum dose of Flumetralin resulted minimum green weight (72.7 g) followed by optimum dose at early flowering stage (99.3). Maximum green weight of suckers (238 g) was recorded in plots where manual de-suckering was done (Table 3). The results were supported by (Hao et al., 2001) who reported that topping stage of tobacco is vital and necessary for key time and improvement of agricultural measures to encourage the quality of leaves and late topping increase the number of pre-topping suckers that must be removed as well as the chance of plants blowing over in a windstorm.

Dry Weight of Sucker plot^{-1}

Analysis of data showed significant disparity of the effect of topping stages with different doses of Flumetralin on the dry weight of sucker plot^{-1}. Lowest dry weight of suckers was found in those plots where topping was done at button stage followed by early stage. The maximum dry weight of sucker plot^{-1} was recorded in control (39.22 g), followed by manual de-suckering in later flowering stage (36.56) and early flowering stage (35.99). Interaction was also founded significant as shown in (Table 4). The dry weight of sucker's plot^{-1} appreciably decreased with an increased in the Flumetralin concentration and treated at button stage of topping compare with control plots. These results were in same channel with the results presented by (Pandeya et al., 2001) who noted that chemical suckericide had important consequence on suckers control and give minimum dry weight of sucker's plant^{-1}.

Cured Weight of Leaves plot (kg)

Topping time and suckericide concentration significantly affected the cured weight of leaves plot^{-1} (Table 5). The highest cured leaves weight (5.51 kg) was recorded in those plots with manual de-suckering, followed by (5.51 kg) with the application of maximum dose and (5.46 kg) with optimum dose of Flumetralin at button stage. The lowest cured weigh was found in control plots. Interaction was also significant at button stage compare with control and late stage of topping presented in (Table 5). The results were also supported by (Wang et al., 2012) who concluded that the aim of topping is to divert the vital nutrients of the plant to the leaves instead of their seeds and flower with the consequences of gaining length and width of the leaf.

Yield (kg ha^{-1})

Analysis of data revealed that the Flumetralin concentration on topping stages influencing yield significantly. The higher yield was founded in plots which were treated by optimum dose of Flumetralin at button stage (3278. kg ha^{-1}) followed by maximum dose of Flumetralin at button stage (3275 kg ha^{-1}).

Table. 5 Cured weight of leaves per plot^{-1} (kg) as affected by manual topping and suckericide application at different stage on FCV tobacco quality and yield.

Treatments	Button stage	Early stage	Late stage	Mean
T0 (control)	5.31	4.71	4.36	4.79 c
T1 (Manual de-suckering)	5.51	4.77	4.44	4.91 b
T2 (Minimum dose)	5.37	4.85	4.4	4.90 b
T3 (Optimum dose)	5.46	4.99	4.56	5.01 a
T4 (Maximum dose)	5.51	5.09	4.63	5.08 a
Mean	5.43 a	4.88 b	4.49 c	

Mean values in the same category, marked by different letters, demonstrate a statistically significant distinction from one another, as evidenced by the LSD test, with a significance level of P<0.05; Least significant difference value for treatments at 0.05 level = 0.075; Least significant difference value for treatments at 0.05 level = 0.058

Table. 6 Yield (kg ha^{-1}) as affected by manual topping and suckericide application at different stage on FCV tobacco quality and yield.

Treatments	Button stage	Early stage	Late stage	Mean
T0 (control)	3189.2	2830	2620	2879.7 e
T1 (Manual de-suckering)	3210	2865	2666.7	2913.8 d
T2 (Minimum dose)	3226.6	2915.7	2684	2942.1 c
T3 (Optimum dose)	3278.3	3000	2741.7	3006.7 b
T4 (Maximum dose)	3275	3059	2780	3038 a
Mean	3235.8 a	2933.9 b	2698.4 c	

Mean values in the same category, marked by different letters, demonstrate a statistically significant distinction from one another, as evidenced by the LSD test, with a significance level of P<0.05; Least significant difference value for treatments at 0.05 level = 16.956; Least significant difference value for treatments at 0.05 level = 13.136

Table. 7 Nicotine (%) as affected by manual topping and suckericide application at different stage on FCV tobacco quality and yield.

Treatments	Button stage	Early stage	Late stage	Mean
T0 (control)	2.34	2.29	2.27	2.29 b
T1 (Manual de-suckering)	2.5	2.27	2.3	2.35 d
T2 (Minimum dose)	2.27	2.25	2.8	2.43 a
T3 (Optimum dose)	2.27	2.2	2.81	2.42 ac
T4 (Maximum dose)	2.03	2.15	2.62	2.26 b
Mean	2.23 b	2.26 b	2.56 a	

Mean values in the same category, marked by different letters, demonstrate a statistically significant distinction from one another, as evidenced by the LSD test, with a significance level of P<0.05; Least significant difference value for treatments at 0.05 level = 0.171; Least significant difference value for treatments at 0.05 level = 0.132

Table. 8 Reducing sugar (%) as affected by manual topping and suckericide application at different stage on FCV tobacco quality and yield.

Treatments	Button stage	Early stage	Late stage	Mean
T0 (control)	7.18	7.4	7.01	7.19d
T1 (Manual de-suckering)	15.45	15.4	15.4	15.41 c
T2 (Minimum dose)	15.44	15.5	15.46	15.47 b
T3 (Optimum dose)	15.44	15.45	15.45	15.44 bc
T4 (Maximum dose)	16.29	16.26	16.19	16.24 a
Mean	13.96 b	14.0 a	13.90 c	

Mean values in the same category, marked by different letters, demonstrate a statistically significant distinction from one another, as evidenced by the LSD test, with a significance level of P<0.05; Least significant difference value for treatments at 0.05 level = 0.045; Least significant difference value for treatments at 0.05 level = 0.034

The lower yield was achieved in check plots in late flowering stage. Interaction of topping time and suckericide concentration were also founded significant. (Wang et al. 2012) reported similar result that to persuade leaf ripening and maximize and courage the leaf production, it is compulsory to control and remove the flower. The aim of removing the flower from the tobacco plant switch off the plant from a reproductive stage to a leaf producing phase.

Nicotine (%)

The lowest nicotine content is an important characteristic for receiving good quality of tobacco leaves. Data regarding nicotine (%) presented in (Table 7) showed that topping time and Flumetralin concentration significantly affected nicotine percentage. The lowest nicotine (%) was founded in those plots which were treated with maximum dose of Flumetralin at button stage (2.03%), followed by control plots with no sprayed of Flumetralin. The highest nicotine (2.81 %) were founded

in plots which were treated with optimum dose of Flumetralin at late flowering stage. The interaction between topping time and concentration were also founded significant. Similar results were also reported by (Mahadevareddy et al. 1990) that the using of 10% ILTD mixture decreased nicotine contents in leaf.

Reducing Sugar (%)

The data about topping and Flumetralin concentration significantly affected reducing sugar (%) in tobacco leaf. The lowest reducing sugar (7.18%, 7.4%, 7.01%) at button, early and late flowering stage respectively, founded in control plot having no sprayed of Flumetralin. Interaction between topping time and Flumetralin concentration indicated that reducing sugar (%) increases with increasing concentration of Flumetralin concentration compared to control plot having no sprayed of Flumetralin. (Mahadevareddy et al. 1990) also reported that 10% ILTD mixture increased nicotine contents and enhanced the reducing sugar contents in tobacco leaf.

Conclusion

Topping in tobacco is one of the most important and vital operations to improve leaf growth, development and quantity. Moreover, application of maximum dose of suckericide; Flumetralin at button stage was more effective in tobacco. The results obtained from the present study indicated that topping at button stage and suckericide concentration (1500 ml ha^{-1}) were more effective for better suckers control, enhanced leaf quality, increases leaf area, better cover of plant, reduced new suckers' growth, minimized green weight of suckers, decreased dry weight of sucker's $plot^{-1}$. The optimum concentration dose (1200 ml ha^{-1}) enhanced nicotine concentration, increased sugar contents, and yield of tobacco crops.

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Comparative Analysis of 1,9-Dimethyl-Methylene Blue and Toluidine Blue Interactions with ct-DNA, G-Quadruplex DNA, and ssDNA

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ABSTRACT

This study presents a comprehensive investigation into the interactions of two distinct synthetic dyes—1,9-Dimethyl-Methylene Blue and Toluidine Blue —with different DNA structures, namely calf thymus DNA (ct-DNA), G-quadruplex DNA, and single stranded DNA (ssDNA). The objective of this research is to elucidate the molecular affinities of these dyes for specific DNA structures and explore their potential applications in molecular biology and diagnostics. The experimental approach involved detailed UV-visible spectroscopic analyses, to probe the binding affinities of each dye with ct-DNA, G-quadruplex DNA, and ssDNA. The study aimed to assess the selectivity of these dyes towards the unique structural features of each DNA entity. The binding stoichiometry is defined from Job's method. The selectivity of the dyes towards DNA also investigated with competitive dialysis experiments. The binding stoichiometry were 1:1 for 1,9-Dimethyl-Methylene Blue and Toluidine Blue and G-quadruplex DNA or ssDNA. Besides, results indicate that 1,9-Dimethyl-Methylene Blue and Toluidine Blue exhibit a pronounced affinity for G-quadruplex DNA, and ct-DNA. While single-stranded DNA is a fundamental component of DNA replication and transcription, our dyes exhibit lower affinity for this structure. The selectivity is advantageous, as it allows for the discrimination between single-stranded and structured DNA regions. The potential applications in studying DNA dynamics and unwinding processes are vast.

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Introduction

Cancer is a complex disease that develops with the accumulation of DNA damage and changes in genome structure and function (Davalos and Esteller, 2023). The cell proliferation increases in cancerous cells. Uncontrolled cell division is one of the most prominent findings in cancer. Due to increased metabolism, DNA replication and transcription are increased in the cancerous cell.

G-quadruplex DNAs are DNA structures that occur in guanine-rich regions of the genome. They have emerged as attractive targets for therapeutic intervention due to their association with critical biological processes, including gene regulation and telomere maintenance (Hirt et al., 2014). These secondary structures are formed by stacking G-tetrads on top of each other. G-tetrads are formed by connecting four guanine bases via Hoogsteen hydrogen bonds. G-quadruplex DNAs become stable with the presence of cations such as sodium or potassium. Stabilization of these structures is important. Because the stabilization of these has been associated with a reduction of telomere ends or a lower oncogene expression (Zegers et al., 2023). In addition, G-quadruplex DNA stabilizing or perturbing ligands have very important potential for use in imaging, theranostic and diagnosis (Holden et al., 2023). Therefore, G-quadruplex DNA is a very important target in the treatment of cancer (Figueiredo et al., 2023).

Small molecules that specifically interact with G-quadruplex structures can modulate their stability and function (Awadasseid et al., 2021). These molecules often possess aromatic moieties and functional groups that facilitate binding to the guanine tetrads (O'Hagan et al., 2019; Pirota et al., 2020). The recognition of G-quadruplexes by small molecules is a highly specific process, guided by factors such as shape complementarity, electrostatic interactions, and hydrogen bonding. The consequences of G-quadruplex DNA-small molecule interactions extend beyond structural stability (Biver et al., 2022; Mendes et al., 2022; Kaushik et al., 2011; Du et al., 2010). These interactions can influence gene expression,

telomere maintenance, and DNA replication. Small molecules that stabilize G-quadruplex structures may act as potential anticancer agents by interfering with telomerase activity or modulating the expression of oncogenes (Takahashi et al., 2021; Lerner and Sale, 2019; Salvati et al., 2007). Ligands with selective affinities hold immense potential for applications in biotechnology and diagnostics (Yang et al., 2010). Their ability to discriminate between various DNA structures makes them valuable tools for designing DNA sensors, detecting specific sequences or structural motifs associated with diseases, and advancing our understanding of molecular interactions within living systems (Li et al., 2010).

Numerous human malignancies, including as osteosarcomas, lymphomas, leukemias, cervical, lung, breast, and prostate cancers, are associated with overexpression of the c-MYC proto-oncogene. High c-MYC expression levels are also frequently linked to a poor outcome for therapy. A variety of processes, including as gene amplification, translocation, and straightforward transcriptional upregulation, can result in c-MYC overexpression (Mathad et al., 2011). Due to these properties, c-MYC234 G-quadruplex structure belonging to the c-MYC promoter region was studied.

1,9-Dimethyl-Methylene Blue and Toluidine Blue are both synthetic dyes that belong to the thiazine dye family. These two dyes are known to interact with DNA. The structural differences contribute to variations in their interactions with DNA. In this study, the interactions of these dyes with ssDNA, G-quadruplex DNA and ct-DNA were examined to understand whether there was a difference in the affinities of these dyes to DNA structures.

Materials and Methods

Double distilled water (dd-H_2O) was used in the preparation of main stock solutions throughout all experiments. pH 7.4 TRIS-HCl buffer solution containing 100 mM KCl was used for dilutions. pH adjustment of the buffer solution was made with a Sartorious basic model pH meter. ct-DNA (Sigma-Aldrich) stock solution was prepared to get a concentration of 1 mg/mL, c-MYC2345 and ssDNA (Alpha DNA, Canada) stock solutions were prepared as 100 micromolar. The solutions were diluted with buffer solution to appropriate concentrations to be used in experiments. c-MYC2345 and ssDNA solutions were incubated at 95ºC for 5 minutes and cooled to room conditions. ct-DNA concentration was calculated from the absorbance value and extinction coefficient of 6600 $M^{-1}cm^{-1}$ (Reichmann et al., 1954).

UV-vis spectra were scanned with a Shimadzu UV 1800 model spectrophotometer and using quartz cuvettes. The stock solutions of 1,9-Dimethyl-Methylene Blue (1,9 DMB) zinc chloride double salt (Sigma-Aldrich) and Toluidine Blue O (TBO) (Sigma-Aldrich) were prepared in dd-H_2O and further diluted with buffer solution. The solution is protected from light.

For the spectrophotometric titration, 8 µM of 1,9 DMB was prepared by diluting the stock solution in pH 7.4 TRIS-HCl buffer solution containing 100 mM KCl. The concentrated DNA (150 µM for ct-DNA, 10 µM for both c-MYC2345 and ssDNA) solution was added in small portions and pipetted several times to mix the solutions homogenously. The absorption spectrum was scanned after each addition and the absorbance values were recorded for each titration point. The control experiment was performed without DNA.

The titrations of TBO were also conducted with the same experimental procedure.

The binding constant, Kb was calculated from (Kocak et al., 2016; Yılmaz et al., 2021):

$$L + DNA \text{ ==== } L\text{-}DNA$$

$$K_b = \frac{[L-DNA]}{[L][DNA]}$$

$$[L]_t = [L] + [L-DNA]$$

Then

$$K_b = \frac{[L-DNA]}{([L]_t - [L-DNA])[DNA]}$$

$$K_b[L-DNA]^2 - \left(1 + K_b[L]_t + \frac{K_b[DNA]}{s}\right)[L-DNA] + \frac{K_b[L]_t[DNA]}{s} = 0$$

$[L\text{-}DNA]/[L]_t$ is the bound fraction and calculated from the absorbance data:

$$A_a = A_f + A_b$$

Where A_a, A_f and A_b is the apparent, free and bound absorption.

$$\varepsilon_a[L]_t = \varepsilon_f[L] + \varepsilon_b[L-DNA] = \varepsilon_f([L]_t - [L-DNA]) + \varepsilon_b[L-DNA]$$

Then

$$\frac{[L-DNA]}{[L]_t} = \frac{\varepsilon_a - \varepsilon_f}{\varepsilon_b - \varepsilon_f}$$

$$\frac{\varepsilon_a - \varepsilon_f}{\varepsilon_b - \varepsilon_f} = \frac{b - \sqrt{(b^2 - \frac{4K_b^2[L]_t[DNA]}{s}}}{2K_b[L]_t}$$

$$b = 1 + K_b[L]_t + \frac{K_b[DNA]}{s}$$

Where εf, εb, and εa are the extinction constants for free, bound dye and apparent mixture of dye, DNA and dye-DNA when both Dye-DNA complex and either one of the dye or DNA is freely available. The Solver tool of excel software (Microsoft) was used for the nonlinear equation solution.

The stoichiometry of binding was determined with Job's method. A series of solutions were prepared where the total concentration (dye+DNA) remains constant, and only the mole fraction of one of the components varies systematically. The absorbance of the solutions was measured as the mole fraction of one component changes. The absorbance against the mole fraction of the dye was plotted.

Table 1. The sequences for c-MYC2345 and ssDNA

c-MYC2345	5'- TGA GGG TGG GGA GGG TGG GGA A
ssDNA	5'- TTC CCC ACC CTC CCC ACC CCT CA

(a) (b)

Figure 1. Chemical strüktürel of a) 1,9-Dimethyl-Methylene Blue and b) Toluidine Blue O.

Competitive Dialysis

A semi-permeable dialyses membrane with a MWCO 3500 (SERVA, Membra-cel, dialysis tubing) that is appropriate for the molecules under investigation was used. 1000 µL of 75 µM of each DNA sample were placed into dialyses membrane. All membranes were placed into a baker which contains 400 mL 1 µM of dye solution. The membrane was allowed for the selective diffusion of the dye molecules while maintaining the competitive aspect of the dialysis. The concentrations of the dyes after dialysis (24 h) was measured spectrophotometrically to assess the competitive interactions (Guittat et al., 2003).

Results and Discussion

UV-Visible Spectrophotometric Titration

UV-Visible spectrophotometric titration is a versatile technique used to investigate molecular interactions by monitoring changes in absorbance as a function of a titrant's concentration. In the context of studying G-quadruplex DNA, this technique allows researchers to observe alterations in the electronic structure of the DNA and the small molecule ligand (Feizi-Dehnayebi et al., 2021). The UV-Visible spectrophotometric titration of small molecules with G-quadruplex DNA is a powerful technique that provides valuable information (such as binding constants) about the binding interactions between the G-quadruplex DNA and small molecule.

The UV-Visible spectrum of 1,9-Dimethyl-Methylene Blue typically exhibits absorption peaks in the visible region of the electromagnetic spectrum. The positions and intensities of these peaks are indicative of the specific electronic transitions occurring within the molecule. The binding of 1,9 DMB to DNA can induce conformational changes in the DNA structure. This interaction may cause alterations in the stacking and arrangement of DNA base pairs. These structural changes can, in turn, influence the electronic interactions between the dye and the DNA, leading to changes in the electronic spectrum. The electronic spectrum of 1,9 DMB typically includes absorption peaks corresponding to specific electronic transitions. The presence of DNA can shift these peaks, broaden them, or induce new peaks, indicating changes in the electronic states of the dye.

As can be seen from Figure 2 and Figure 3, 1,9 DMB and TBO have a large absorption band between 550-700 nm range. The addition of DNA to dye resulted in hypochromic effect on these band. Besides hypochromic effect, the wavelength shift also observed. Especially for 1,9 DMB, the interaction with ct-DNA and c-MYC2345 resulted in a significant hypsochromic effect.

The binding constant, also known as the association constant (K_b), quantifies the strength of the interaction between two molecules, typically a ligand and a receptor. The K_b values for 1,9 DMB were $(1.09\pm0.02)x10^7$, $(1.67\pm0.18)x10^6$, and $(1.89\pm0.83)x10^7$ M for c-MYC2345, ssDNA and ct-DNA, respectively. As can be seen, the K_b values for c-MYC2345 and ct-DNA were found ten times higher than Kb of ssDNA. The K_b values for TBO were $(5.75\pm4.25)x10^6$, $(2.11\pm1.18)x10^6$, and $(1.82\pm1.46)x10^7$ M for c-MYC2345, ssDNA and ct-DNA, respectively. The affinity of TBO to ct-DNA was found higher.

The determination of binding stoichiometry for small molecule-DNA is important to evaulate the mechanism. The small molecule typically has functional groups that can form specific interactions with the G-quadruplex structure. These interactions may include hydrogen bonding, π-stacking, electrostatic interactions, and van der Waals forces (Chen et al., 2013). The binding stoichiometries of dyes with c-MYC2345, ssDNA were evaluated using Job's method. As shown Figure 4 - Figure 5 the binding stoichiometries were found 1:1 for all circumstances. The mechanism of 1:1 G-quadruplex-small molecule interaction can vary depending on the nature of the small molecule and the specific G-quadruplex structure involved. The 1:1 binding stoichiometry may be due to end stacking. End-stacking refers to the interaction of aromatic moieties at the termini of adjacent G-tetrads in a G-quadruplex. This phenomenon plays a pivotal role in stabilizing the G-quadruplex structure, contributing to its overall stability. The aromatic rings, often derived from guanine bases, engage in π - π stacking interactions, creating a continuous stacking interface at the ends of the G-tetrads. End-stacking interactions not only enhance the thermal stability of the G-quadruplex but also influence its folding topology. It's important to note that the actual mechanism can be highly specific and may involve multiple steps and factors. Additionally, experimental techniques such as NMR spectroscopy, X-ray crystallography, or molecular modeling needed often employed to elucidate the details of the binding mechanism and the structural changes that occur upon interaction.

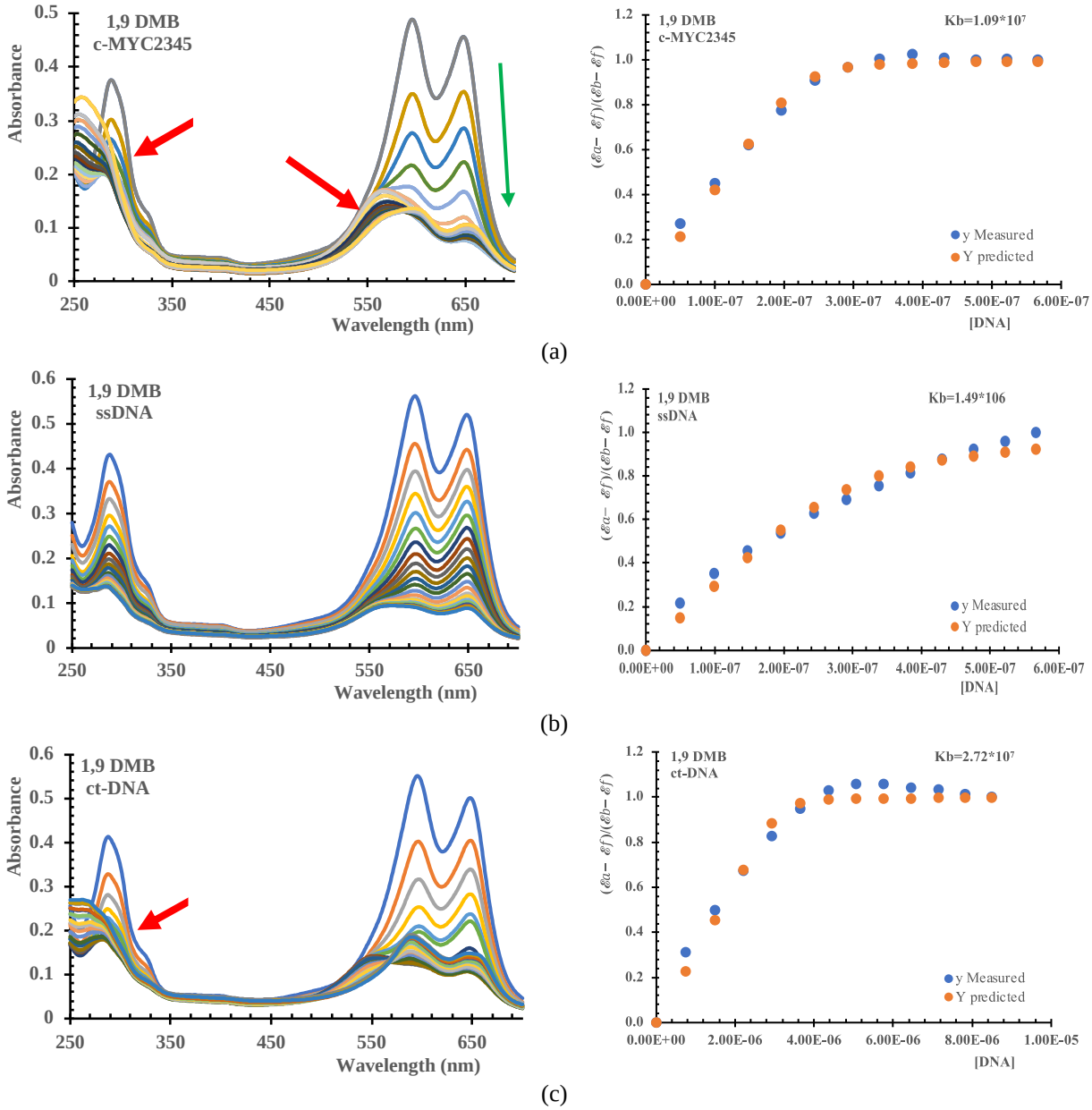

Figure 2. UV-Visible titration spectrum and binding curve for 1,9 DMB (8 µM) with c-MYC2345, ssDNA and ct-DNA (in pH 7.4 TRIS-HCl, 100 mM KCl, unit of DNA concentration and Kbs are M)

Competitive Dialysis Experiments

In biochemical research, competitive dialysis is used to explore interactions between biomolecules, such as nucleic acids, enzymes, and small molecules (Ren and Chaires, 1999). This aids in unraveling the complex networks of molecular associations within living systems. Competitive dialysis takes the principles of traditional dialysis a step further by introducing a competitive element into the equation. This technique involves the co-dialysis of two or more molecules through a semi-permeable membrane, where they compete for binding sites or interactions. The relative affinities of these molecules for the binding sites on the membrane or for each other can be studied, providing valuable insights into the dynamics of molecular interactions.

Competitive dialysis is a technique employed to study the relative affinities of a molecule, in this case, a dye, for different DNA structures. This method involves setting up a dynamic equilibrium where the dye competes for binding sites on various DNA structures in a controlled environment. The dialysis membrane allows for the exchange of small molecules while retaining the larger DNA structures, enabling the quantification of binding affinities. As the experiment progresses, the concentrations of the dye in each membrane reach an equilibrium based on their respective affinities for the DNA structures. By monitoring the concentrations over time, we can derive information about the relative binding affinities of the dye for single-stranded DNA, G-quadruplex, and calf thymus DNA.

As can be evaluated from Figure 6, the affinities of 1,9 DMB and TBO to c-MYC2345 and ct-DNA are higher. The equilibrium concentration of TBO for c-MYC2345 was 35.28 µM which is the highest value for competitive dialysis experiment.

Conclusion

When all experimental results were evaluated together, it was observed that both dye molecules showed higher affinity for ct-DNA and c-MYC2345 DNA. Although the experiments did not provide information about the binding mechanism, basic information about binding stoichiometry, binding constants and relative affinities was obtained. This study is a basic study in which the interactions are determined, and further studies are needed to determine the binding mechanism and thermodynamic data on binding.

The study of G-quadruplex-small molecule interactions is a dynamic field, and researchers continue to explore and understand the intricacies of these molecular interactions for potential therapeutic applications. While the promise of G-quadruplex DNA-small molecule interactions in drug development is evident, challenges such as cellular uptake and specificity remain. Advances in structural biology techniques, including X-ray crystallography and NMR spectroscopy, have provided invaluable insights into the detailed interactions at the molecular level, aiding in the rational design of small molecules with enhanced binding affinity and selectivity.

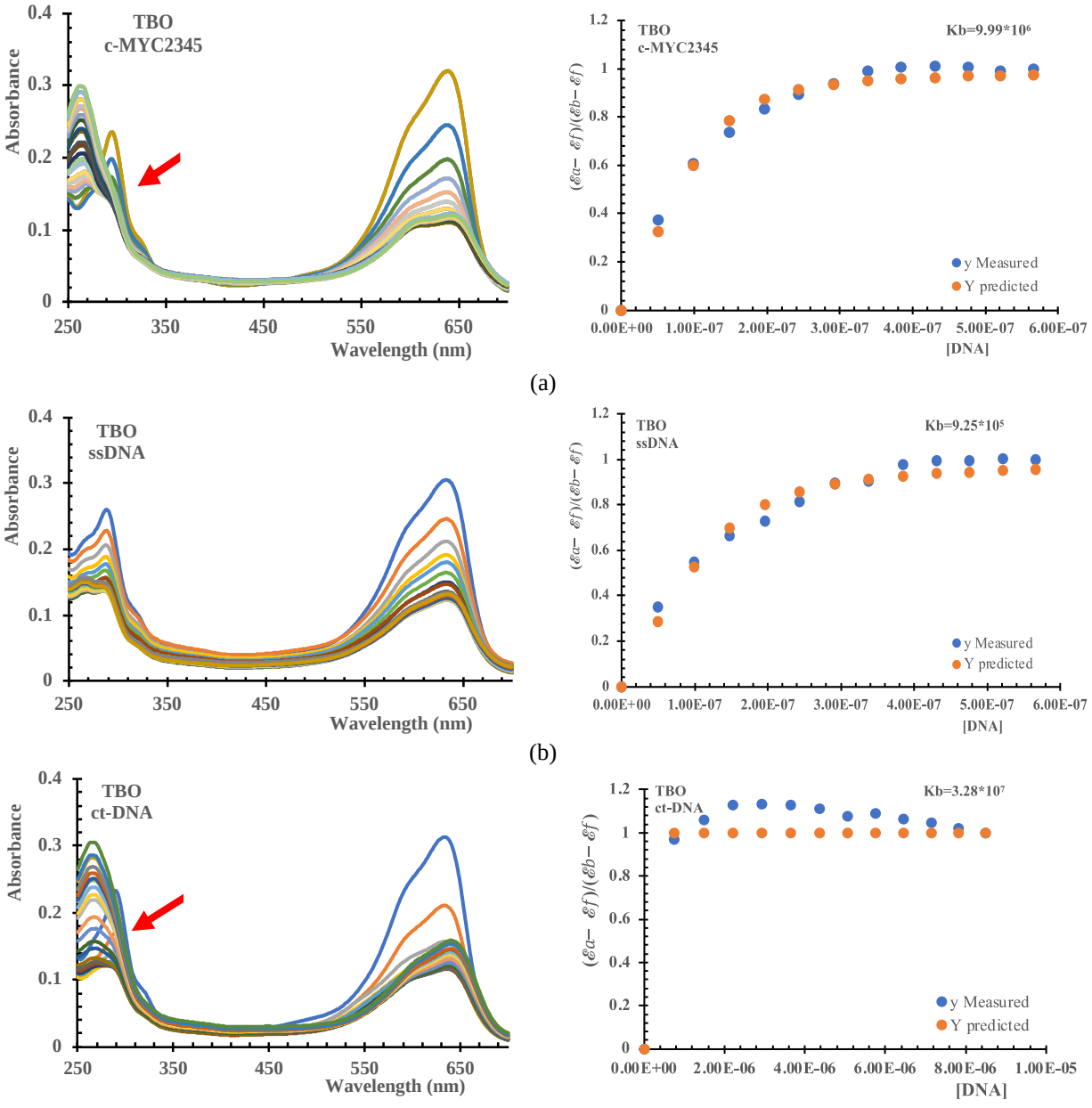

Figure 3. UV-Visible titration spectrum and binding curve for TBO (8 µM) with c-MYC2345, ssDNA and ct-DNA (in pH 7.4 TRIS-HCl, 100 mM KCl).

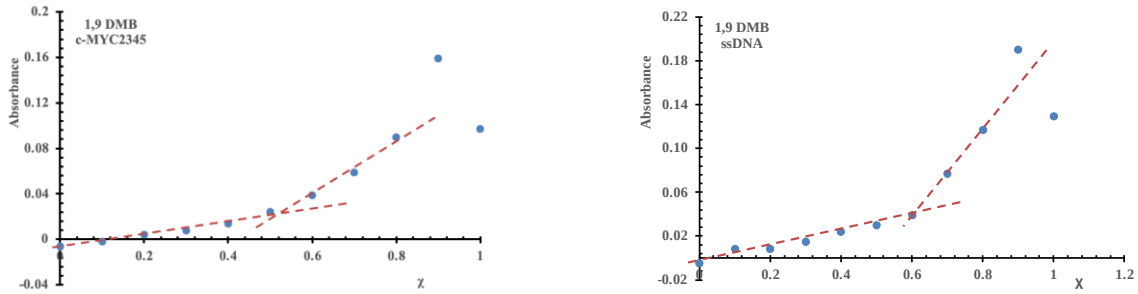

Figure 4. Job's Plot for 1,9 DMB with c-MYC2345, ssDNA and ct-DNA (in pH 7.4 TRIS-HCl, 100 mM KCl).

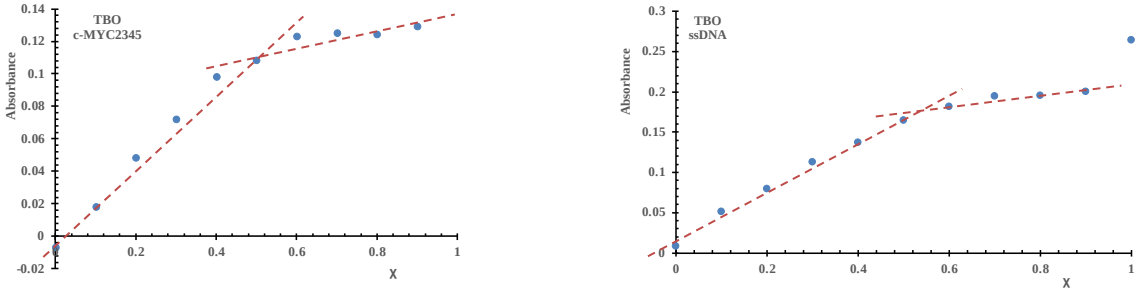

Figure 5. Job's Plot for TBO with c-MYC2345, ssDNA and ct-DNA (in pH 7.4 TRIS-HCl, 100 mM KCl).

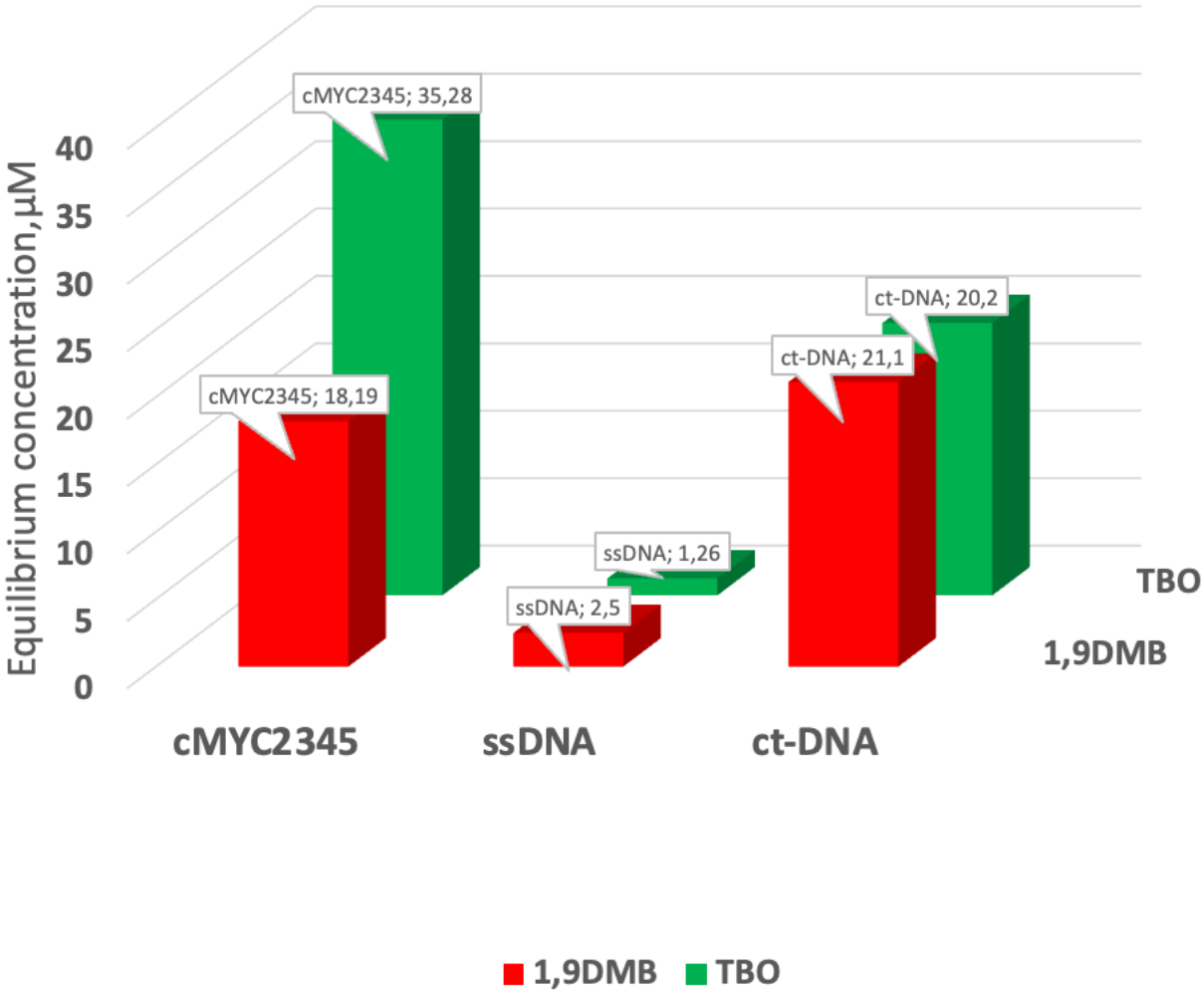

Figure 6. Competitive dialysis

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Morphological and Physiological Responses of Different Cotton Genotypes Primed with Salicylic Acid Under Salinity Conditions

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ABSTRACT

This study was conducted as both petri dishes and pot experiments on four different salt-sensitive cotton genotypes (Laser, May 505, May 455 and Selin) in order to investigate the role of exogenous salicylic acid applications in reducing the effects of salt stress. Six saline treatments; 0, 30, 60, 90, 120 mM NaCl were used. Each group divided into three sub-groups (hydo-primed control, 0.5 mM and 1.0 mM SA) on the basis of seed priming treatments. They were applied in three replications according to the randomized block design. In all genotypes, 90 mM and 120 mM salt stress negatively affected germination and seedling development. In salt stress up to 60 mM, it was recommended to May 505 and Selin genotypes with 0.5 mM salicylic acid pre-application to the seeds.

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Introduction

Cotton is considered a salt-tolerant crop after barley, with a salt stress threshold level of 7.7 dS m^{-1}, with moderate tolerance to salt stress (Alizade & Mammodova, 2023; Muhammad et al., 2023). The roots of the cotton are the first organs to be affected by salt and root development (Öz & Karasu, 2007), growth, yield and fiber quality are adversely affected.

Salt stress is the second most common abiotic stress after drought, negatively affecting plant growth and significantly limiting plant yield (Anwar et al., 2023). Salty and alkaline soils constitute more than 6% of the world's soils. Seed germination is affected by adverse environmental conditions, including salinity (Abdi et al., 2022). Salt stress causes a decrease in biomass, stem thickness, leaf area, root and shoot weight and seed yield in cotton (Sharif et al., 2019) and stimulates antioxidant enzymes such as superoxide dismutase, peroxidase, catalase and glutathione reductase (Alizade & Mammodova, 2023; Muhammad et al., 2023), on the other hand, it tends to decrease as a result of increasing salicylic acid (SA) level (Abdi et al., 2022).

In conditions of intense salt stress, seeds cannot absorb sufficient water due to higher osmotic potential, which reduces or delays the germination process in seeds (Azeem et al., 2019). Salt stress causes a significant decrease in leaf chlorophyll index (Demming & Adams, 1996) and photosynthesis rate, which is one of the main regions in photosynthesis and is directly related to photosynthesis performance (Harizanova & Koleva-Valkova, 2019).

Germination is the most sensitive stage in the life cycle of a plant and salt-induced stress inhibits the germination and development of seeds (Biswas et al., 2023; Radwan et al., 2023; Taşan, 2023). Salinity affects germination negatively in cotton (Malik et al., 1994; Amjad et al., 2002; Munawar et al., 2021) and as the salt dose increases biomass, root length and root surface area decrease. High salt concentration prevents germination and seedling growth in cotton (Yan et al., 2019). Salt stress reduces plant height in cotton (Shahzad et al., 2020), reduces germination rate (Ergin et al., 2021), biomass (Guo et al., 2019), negatively affects growth (Long et al., 2019; Ergin et al., 2021; Hamani et al., 2021).

One of the methods used to alleviate the effects of salt stress is seed priming (Fujikura et al., 1993; Radwan et al., 2023). This technique provides rapid and uniform seedling emergence, plays a role in breaking seed dormancy, increases protein synthesis, plant growth and development by providing resistance to environmental stress factors (Moreno et al., 2018; Moghaddam et al., 2020; Anwar et al., 2023).

Salicylic acid a phenolic phytohormone, causes many metabolic and biochemical changes in germinating seeds (Farooq et al., 2013), regulates growth with various physiological responses and also plays an important role in reducing the effects of temperature, salt, osmotic and oxidative stress (Khan et al., 2012; Riaz et al., 2019; Sofy et al., 2020; Abdi et al., 2022; Biswas et al., 2023; Maqsood et al., 2023; Ogunsiji et al., 2023). It significantly increases salinity tolerance based on glycine betaine to saline conditions, followed by an increase in water content. While salinity stimulates antioxidant enzymes such as superoxide dismutase, peroxidase, catalase and glutathione reductase, it tends to decrease as a result of increasing salicylic acid level (Abdi et al., 2022).

Salicylic acid has a positive effect on growth, yield and quality characteristics of cotton (Al-Rawi et al., 2014), strawberry (Lolaei et al., 2012) and tomato (Yıldırım & Dursun, 2009). Salicylic acid increases plant tolerance to stress conditions by balancing the decrease in dry weight in plants. This is due to the stimulating effect of salicylic acid on shoot growth and the accumulation of more assimilates in the shoots (Pirasteh-Anosheh et al., 2014). With the application of SA to wheat and maize, abscisic acid and indole acetic acid accumulate (Fahad et al., 2015). Salinity reduces fresh weight (40%) and chlorophyll (39%) in wheat, whereas root fresh weight and chlorophyll b increase after 20 mM SA priming to seeds (Maqsood et al., 2023). Salicylic acid increases cell membrane damage caused by salt stress, thus reducing the transpiration rate, facilitating the adjustment of the optimum amount of water in plant tissues and minimizing water loss (Fairoj et al., 2022).

The application of phytohormones such as salicylic acid will be effective in mitigating or minimizing the negative impact of salinity on plant growth and productivity (Moles et al., 2019).

Therefore, it was aimed to determine the effect of salicylic acid priming on seed germination, seedling growth, morphological and physiological parameters in four cotton genotypes grown under salinity stress in this study. The hypothesis is that salicylic acid application may increase the salinity tolerance of cotton genotypes during germination and seedling periods.

Materials and Methods

Location of the Experiment

The study was carried out at Ege University, Faculty of Agriculture, Department of Field Crops and Aydın Nazilli Cotton Research Institute.

Plant Material

The characteristics of four different cotton genotypes used (Laser, May 505, May 455 and Selin) in the experiment are shown in Table 1.

Soil Properties

The properties of the soil used in the experiment are given in Table 2. This soil used was mixed with peat at a ratio of 1:1 and added to each pot equally. A total of 216 pots were used.

According to the soil analyzes, it was determined that the soil texture is sandy-loamy, moderately alkaline, poor in $CaCO_3$ ($CaCO_3$ %<2.5) and there is no salinity problem (total salt %< 0.150).

Treatments and Experimental Design

A completely randomized design was adopted with the levels of different salinity doses (0; 30; 60; 90; 120 mM) and salicylic acid concentrations (0; 0.5; 1.0 mM).

Germination tests were carried out in the growth incubating chamber (28 ± 1°C) in Aydın Nazilli Cotton Research Institute Laboratory, with a diameter of 9 cm and a depth of 2 cm (0.18 lt) petri dishes placed with double filter paper and 15 seeds on them. Pot experiments were carried out in growth incubating chamber, 15 cm diameter and 20 cm deep pots (3 L) in Aydın Nazilli Cotton Research Institute Laboratory.

Table 1. Characteristics of cotton genotypes used in the experiment

Properties	Genotypes			
	Lazer	Selin	May455	May505
100 grain weight (g)	7.78	8.43	11.01	9.59
Fiber fineness (mikroinere)	4.6 - 4.8	4.7	4.4 - 4.8	4 - 4.6
Fiber strength (g/tex)	34 - 36	31.5	32 - 35	32 - 36
Fiber length (mm)	31 - 32	28.7	30 - 31	30
SCI	160 - 180	-	135	135
Boll opening rate	Medium	Late	Early	Early
Average seed cotton yield ($kg.ha^{-1}$)	58.4	54.8	56.2	41.6
Ginning outturn (%)	45 - 47	42.8	44 - 46	41- 43
Fiber yield ($kg.ha^{-1}$)	26.52	23.46	24.15	20.13

Table 2. Properties of the soil used in the experiment

pH	NaCl (%)	$CaCO_3$ (%)	Sand (%)	Silt (%)	Clay (%)	P ($mg.kg^{-1}$)	K ($mg.kg^{-1}$)
7.9	0.15	2.04	65.7	26.9	7.4	2.65	145
	Na ($mg.kg^{-1}$)	Fe ($mg.kg^{-1}$)	Ca ($mg.kg^{-1}$)	Mg ($mg.kg^{-1}$)	Zn ($mg.kg^{-1}$)	Cu ($mg.kg^{-1}$)	S ($mg.kg^{-1}$)
	118	7.09	2230	457	0.38	1.12	120.4

Seed Priming

Cotton seeds were surface sterilized with 1% sodium hypochlorite (NaOCl) for 2 minutes and then thoroughly washed with sterilized water (Azeem et al., 2019). Seeds were soaked either in water (hydro-priming) or 0.5 and 1.0 mM SA solutions (SA priming), for 12 h.

Seed Germination in Laboratory

Healthy and uniform seeds from primed treatments were transferred into petri plates (15 seeds per plate), lined with double layer of filter paper. Petri plates were divided into five groups, moistened with 10 ml of 1) distilled water (non-saline control), 2) 30 mM NaCl solution, 3) 60 mM NaCl solution, 4) 90 mM NaCl solution and 5) 120 mM NaCl solution. Each group was further divided into three treatments, 1) hydro-priming 2) primed with 0.5 mM SA and 3) primed with 1.0 mM SA. Each treatment had 3 replicates. Petri-plates were placed in growth incubating chamber at 28 ± 1ºC, (12 h light and 12 h dark) for 14 days. A total of 216 petri plates were used. The protuberance of radical (2 mm) was considered as a mark of germination (Mohammadi, 2009). After 14 days, seedlings (3 of each replicates) were kept in oven (70ºC for 48 h) for dry weight measurements. The plumule and radicle lengths, fresh weight, dry weight and chlorophyl content index (SPAD) were measured.

Pot experiment

Fifteen seeds of three priming treatments (0, 0.5 and 1 mM SA) were sown in 3 L plastic pots containing (15-cm diameter at the top and 20-cm depth) filled with 2.8 kg of soil and peat at 1:1 ratio. By using 15.10.10 compound fertilizer, 15 kg of pure nitrogen, 10 kg of pure phosphorus and 10 kg of pure potassium were fertilized per decare. Pots were placed in climate laboratory with 6 saline treatments, 1) 0 mM (non-saline), 2) 30 mM NaCl, 3) 60 mM NaCl, 4) 90 mM NaCl and 5) 120 mM NaCl were used. Each group divided into three sub-groups on the basis of seed priming treatments 1) hydo-primed control, 2) 0.5 mM SA and 3) 1.0 mM SA. All pots were placed according to completely block design. Pots were observed every day from the start of the study and thinned 28 days after the emergence of seeds.

At the end of four weeks, the plants were harvested and observations were taken. Root length (cm), shoot length (cm), root, shoot fresh and dry weights (g) and chlorophyl content index (SPAD) were evaluated in the seedlings.

Results and Discussions

Labaratory Petri Plates Analyses

The results of the analysis of variance for the observations measured 14 days after sowing of cotton genotypes in which different salt doses were applied under laboratory conditions are summarized in Table 3. The interaction of genotype x salicylic acid x salinity was found to be statistically significant in other properties except plumula and radicle length. Besides, salicylic acid x salinity interaction was found to be significant for plumula length and genotype x salicylic acid interaction for radicle length.

Germination Percentage (%) and Chlorophyll Content Index (SPAD)

The germination percentage of Selin genotype was the highest (100%) under control conditions, followed by Lazer genotype (80%) (Table 4). However, in both genotype, it was observed that the germination percentage was negatively affected by salicylic acid application under salt stress conditions. In general, for all genotypes, the germination (%) decreased at 90 mM salt stress. Although the Selin genotype had the highest germination rate, salicylic acid application could not prevent the decrease in germination as the salt stress increased.

Azeem et al. (2019) emphasized that as salt stress increases in wheat, germination rate is inhibited, and seed priming process significantly reduces seed dormancy. Salicylic acid application to broad bean seeds against salt stress improved germination (Anaya et al., 2018). A positive effect of 20 mM dose of salicylic acid on seed germination rate was observed (Heidarian & Roshandel, 2021). In another study, salicylic acid (10 mM) increased germination percentage in black bean under salt stress (50 mM and 100 mM) conditions. To reduce the adverse effects of salinity on seed germination and plant performance of *Citrullus lanatus*, priming with the pre-sowing seaweed *Ulva lactuca* improved germination and seedling growth (Radwan et al., 2023). In a study conducted on wheat, it was observed that salicylic acid application against salinity increased germination and seedling growth (Shakirova, 2007).

Under salt stress conditions, the effect of salicylic acid applications on SPAD values of genotypes was found to be significant (Table 4). The genotype with the highest SPAD value was Laser.

Table 3. Analysis of the variance of the data of petri plates observations of cotton genotypes

Sources of Variation	DF	Mean Square Values					
		GP	PL	RL	FW	DW	CCI
Genotype (A)	3	33.79 **	177.73 **	416.72ns	12.559 **	0.369 **	289.46 **
Salicylic acid (B)	2	26.39 **	88.29 ns	89.38ns	11.059 **	0.273 **	207.67 **
Salinity (C)	5	66.06 **	95.55 *	226.57 **	2.595 **	0.074 **	84.41 **
A×C	15	25.54 **	41.84ns	86.79ns	0.764ns	0.066 **	88.86 **
A×B	6	4.48ns	26.72 ns	253.43 **	0.519ns	0.021ns	121.07 **
B×C	10	32.99 **	97.71 *	64.81ns	1.529 *	0.078 **	90.91 **
A×B×C	30	24.89 **	33.52ns	57.71ns	1.180 **	0.063 **	73.86 **
Error	142	2.67	40.49	65.56	0.678	0.019	5.63

GP: Germination percentage; PL: Plumule length; RL: Radicle length; FW: Fresh weight; DW: Dry weight; CCI: Chlorohyll content index (SPAD); * and ** indicate significance at 0.05 and 0.01 levels of probability, respectively. ns; not significant;

Table 4. Effect of seed priming of salicylic acid on germination percentage and SPAD value of cotton genotypes

SA (mM)	Salinity (mM)	Germination percentage (%)				Chlorophyll content index (SPAD)			
		Lazer	Selin	May 455	May 505	Lazer	Selin	May 455	May 505
0	0	80.0b	82.2a	66.6d	71.1c	33.53a	23.53b	32.38a	24.93b
	30	64.4a	24.4d	60.0b	62.2a	32.30a	22.94b	33.84a	19.71b
	50	37.7b	28.8d	33.3c	62.2a	30.53a	20.06bc	16.38c	23.62b
	60	28.8d	62.2b	73.3a	53.3c	30.18a	30.65a	26.94ab	23.47b
	90	24.4d	53.3c	60.0a	55.5b	30.13ab	26.36b	33.10a	18.92c
	120	77.7b	100.0a	48.8d	75.5c	30.33a	23.63b	25.98b	27.35ab
0.5	0	68.8a	53.3c	53.3c	64.4b	39.37a	23.39c	23.35c	27.69b
	30	73.3b	84.4a	20.0d	66.6c	19.30b	22.57ab	12.73c	24.66a
	50	53.3d	75.5a	62.2c	68.8b	33.54a	21.33c	19.51c	25.31b
	60	51.1c	37.7d	60.0a	57.7b	33.87a	16.85c	29.35b	30.89ab
	90	22.2d	75.5a	46.6b	33.3c	17.99b	24.13a	17.81b	20.61ab
	120	17.7c	28.8b	26.6b	75.5a	18.94bc	22.43b	17.75c	32.35a
1.0	0	46.6b	26.6d	42.2c	68.8a	26.94a	17.97b	21.50b	20.69b
	30	62.2b	62.2b	64.4a	28.8c	29.86a	20.63bc	23.29b	16.89c
	50	73.3a	28.8d	57.7b	35.5c	33.63a	27.22b	23.27c	21.23d
	60	26.6d	55.5a	33.3c	48.8b	29.24a	25.80a	20.27b	21.16b
	90	24.4c	68.8a	11.1d	55.5b	19.73b	22.57b	14.07c	28.37a
	120	15.5d	53.3a	40.0c	42.2b	17.77c	24.06b	34.19a	25.38b
		$LSD_{(A\times B\times C)}$ = 2.652				$LSD_{(A\times B\times C)}$= 3.850			

Table 5. Effect of seed priming of salicylic acid on plumule length of cotton

SA (mM)	Salinity (mM)	Plumule length (mm)			
		Lazer	Selin	May 455	May 505
0	0	23.70a	27.30a	23.70a	23.30a
	30	26.00a	26.87a	28.88a	27.18a
	50	26.14a	25.00a	18.33a	24.14a
	60	25.39a	27.79a	21.98a	23.37a
	90	17.79a	21.90a	16.91a	24.26a
	120	23.67a	27.28a	27.37a	23.28a
0.5	0	21.83a	21.83a	21.04a	24.83a
	30	16.10b	25.20a	14.40b	21.40a
	50	31.87a	21.95a	29.58a	25.98a
	60	20.33b	27.67ab	26.49b	37.60a
	90	17.77a	22.83a	20.85a	25.07a
	120	11.36b	20.83ab	22.92a	22.60a
1.0	0	17.15a	17.67a	23.46a	21.67a
	30	18.33a	18.51a	27.10a	18.10a
	50	19.40a	21.87a	22.32a	24.33a
	60	20.33a	25.95a	20.83a	25.05a
	90	19.37a	20.70a	14.83a	24.83a
	120	20.0a	25.75a	26.04a	27.71a
		$LSD_{(B\times C)}$= 10.322			

Application of 0.5 mM salicylic acid under control conditions increased all genotypes except May 455 genotype. However, the SPAD value decreased with increasing salicylic acid dose. In a study conducted under salt stress conditions in wheat, it was found that chlorophyll content, tillering number and K^+/Na^+ ratio decreased, but there was a significant improvement after salicylic acid application (Suhaib et al., 2018).

Plumule Length (cm)

The most important parameters in the sensitivity of seeds to salinity are root and plumule length (Jamil et al., 2006). Plumula length was significantly affected by salicylic acid and salt applications (Table 5). However, difference was not significant between genotypes in terms of plumule lengths. *Lathyrus sativus* L. seeds primed with salicylic acid gave longer radicles and plumules length, regardless of the salicylic acid dose. Salicylic acid application under salt stress increased the fresh and dry weight of *Lathyrus sativus* L. seedlings. The effect on seedling dry weight was observed only at a dose of 0.2 mM salicylic acid (Moghaddam et al., 2020). In another study, salt stress adversely affected germination, root length, plumule length, root fresh weight, plumule fresh weight and mean germination time in canola. However, in canola primed with ascorbic acid, it was observed that ascorbic acid alleviated the negative effect of salt on these properties (Taşan, 2023).

Radicle length (cm)

The Lazer genotype under control condition gave the highest radicle length with 0.5 mM and 1.0 mM salicylic acid doses (Table 6). Delavari et al. (2014) reported that due to salicylic acid application to *Ocimum basilicum* under salt stress, osmotic stress decreased and better water uptake was achieved, germination, root and shoot length, fresh and dry weight increased. In saline conditions, soaking bell pepper with salicylic acid improved relative moisture content, radicle and seedling length, dry weight, and vigor, mitigating the toxic effects of salt on the plant (Júnior et al., 2020). Similarly, as the salt dose increased, the decrease in *Lathyrus sativus* plumula and seedling length was greater. Salinity and priming with salicylic acid affected the radicle, plumule and seedling length of *Lathyrus sativus*, but it turned out that the observed effect was not dependent on the dose of salicylic acid (Moghaddam et al., 2020).

Fresh and Dry Weight (g)

The highest fresh weight value of seeds germinated in petri dishes was obtained in the Selin genotype with 3.78 g under control conditions (Table 7). After salicylic acid application (0.5 mM) under salt stress conditions (50 mM), May 505 variety ranked second with 3.60 g. As salt stress increased, a decrease was observed in the Selin genotype fresh weight value, while a high decrease was not detected in the May 505 genotype. In addition, at the highest salt dose (120 mM), fresh weights increased as the salicylic acid dose increased in all cultivars. Priming the seeds with salicylic acid against salt stress causes the plants to accumulate abscisic acid for adaptation.

Abscisic acid promotes various anti-stress proteins that protect plants against stress conditions (Pirasteh-Anosheh et al., 2014; Heidarian & Roshandel, 2021). The cultivar with the highest dry weight was Selin genotype with 0.79 g under the conditions of 120 mM salt application and no salicylic acid (Table 7). Following this, under 120 mM NaCl conditions, May 505 genotype (0.70 g) without salicylic acid gave the highest value, followed by Selin genotype. May 505 genotype gave generally higher values compared to other genotypes when salicylic acid and NaCl doses were taken into account. A study conducted revealed that salt stress significantly reduced seedling dry weight and priming with 0.2 mM salicylic acid increased (Moghaddam et al., 2020). External salicylic acid application to the plant improved fresh weight, dry weight, leaf number (Hayat et al., 2005) and leaf area (Khan et al., 2003a). Treatment of wheat seeds with salicylic acid increased the seedlings' tolerance to salt (Hamada & Al-Hakimi, 2001; Shakirova et al., 2003). There was a significant decrease in growth parameters in cumin under severe salt stress (50 mM NaCl), but application of Amla extract to cumin seeds before sowing improved plant height, number of branches, fresh weight, number of seeds and seed weight, and photosynthetic pigments (Said & Mohammed, 2023).

Pot Experiment Results

Root and shoot related values and chlorophyll content index (SPAD) were significantly affected by the genotype × salicylic acid × salinity interaction (Table 8, Table 9).

Table 6. Seed priming of salicylic acid regulates the radicle length of cotton

SA (mM)	Radicle length (mm)			
	Lazer	Selin	May 455	May 505
0	34.95 a	20.49 b	23.04 b	23.84 b
0.5	25.77 ab	20.99 b	23.35 ab	27.14 a
1.0	24.59 a	24.75 a	23.14 a	20.93 a
	$LSD_{(A\times B)}$= 5.362			

Table 7. Effect of seed priming of salicylic acid on fresh and dry weight of cotton

SA (mM)	Salinity (mM)	Fresh weight (g)				Dry weight (g)			
		Lazer	Selin	May 455	May 505	Lazer	Selin	May 455	May 505
0	0	1.52b	3.78a	2.95a	2.57a	0.43a	0.63a	0.56a	0.58a
	30	1.76ab	0.98a	2.40a	2.26ab	0.41ab	0.21b	0.52a	0.50a
	50	2.18ab	1.78b	0.94b	2.52a	0.48a	0.25b	0.26ab	0.41ab
	60	0.70b	2.04a	2.91a	2.66a	0.20b	0.53a	0.68a	0.62a
	90	0.67b	1.22ab	1.63ab	2.11a	0.22b	0.37ab	0.53a	0.46a
	120	1.38b	3.14a	1.95ab	2.78a	0.42c	0.79a	0.54bc	0.70ab
0.5	0	1.23a	1.90a	2.07a	2.43a	0.40a	0.45a	0.49a	0.49a
	30	1.19ab	2.29a	0.43b	1.93a	0.38a	0.54a	0.12b	0.54a
	50	1.19c	2.07bc	2.84ab	3.60a	0.36b	0.50ab	0.55ab	0.63a
	60	0.99b	1.11b	1.77ab	3.07a	0.22b	0.31ab	0.46a	0.52a
	90	0.59a	1.50a	1.32a	1.29a	0.14b	0.48a	0.30ab	0.26ab
	120	0.30b	0.86b	0.94b	2.57a	0.06b	0.17b	0.24b	0.67a
1.0	0	0.98ab	0.68b	0.41ab	2.07a	0.27b	0.20b	0.51a	0.57a
	30	1.03a	1.24a	1.13a	0.87a	0.35ab	0.37ab	0.43a	0.17b
	50	1.22a	0.85a	1.65a	1.71a	0.47a	0.22b	0.54a	0.34ab
	60	0.60a	1.70a	0.96a	1.38a	0.16b	0.46a	0.32ab	0.43a
	90	0.57ab	1.51ab	0.31b	1.77a	0.14b	0.50a	0.08b	0.46a
	120	0.43b	1.43ab	1.73ab	2.20a	0.10b	0.47a	0.43a	0.43a
	$LSD_{(A\times B\times C)}$= 1.335					$LSD_{(A\times B\times C)}$= 0.225			

Table 8. Analysis of the variance of the root data of pot experiments of cotton genotypes

Sources of Variation	DF	Mean Square Values		
		Root length	Root fresh weight	Root dry weight
Genotype (A)	3	13.39 **	0.16 **	0.018 **
Salicylic acid (B)	2	73.55 **	0.22 **	0.014 **
Salinity (C)	5	422.39 **	0.83 **	0.007 **
A×C	15	92.00 **	0.17 **	0.033 **
A×B	6	55.34 **	0.21 **	0.010 **
B×C	10	153.80 **	0.26 **	0.011 **
A×B×C	30	102.66 **	0.19 **	0.011 **
Error	142	0.09	0.00	0.00

* and ** indicate significance at 0.05 and 0.01 levels of probability, respectively. ns; not significant

Table 9. Analysis of the variance of the shoot data of pot experiments of cotton genotypes

Sources of Variation	DF	Mean Square Values			
		Shoot length	Shoot fresh weight	Shoot dry weight	Chloroyll content index (SPAD)
Genotype (A)	3	285.48 **	2.37 **	0.023 **	774.81 **
Salicylic acid (B)	2	69.77 **	0.19 **	0.094 **	781.81 **
Salinity (C)	5	1010.90 **	37.55 **	0.433 **	702.99 **
A×C	15	56.27 **	5.68 **	0.047 **	138.98 **
A×B	6	36.30 **	9.69 **	0.093 **	171.59 **
B×C	10	56.93 **	5.09 **	0.043 **	309.78 **
A×B×C	30	34.25 **	6.21 **	0.048 **	128.19 **
Error	142	0.08	0.00	0.00	5.96

Table 10 Effect of seed priming of salicylic acid on root and shoot length of cotton genotypes

SA	Salinity	Root length (cm)				Shoot length (cm)			
(mM)	(mM)	Lazer	Selin	May 455	May 505	Lazer	Selin	May 455	May 505
0	0	9.53d	10.3c	23.53a	14.50b	18.38d	28.50a	19.43c	23.87b
	30	20.60a	15.47c	17.83b	21.10a	18.33d	27.93a	20.00c	21.93b
	50	16.60b	3.33c	19.33a	16.17b	17.70b	4.33c	17.53b	23.27a
	60	11.33d	19.73c	23.47b	30.93a	17.20b	17.43b	19.40a	15.50c
	90	21.93b	25.77a	15.37d	17.93c	10.60c	18.53a	18.00b	18.27ab
	120	4.30b	3.67b	3.67b	3.33b	5.27a	4.67b	4.67b	4.33b
0.5	0	13.93b	14.77a	14.27a	12.20c	16.93d	22.80a	22.13b	21.20c
	30	11.33c	27.30a	19.20b	11.50c	21.83a	20.83b	20.40b	20.57b
	50	29.30a	19.50b	19.33b	14.20c	18.07c	16.90d	21.23a	19.83b
	60	27.30a	15.27c	27.30a	16.83b	13.27c	16.77a	16.23b	17.17a
	90	9.30d	22.87a	11.43c	20.93b	7.83d	13.03b	12.23c	18.03a
	120	17.27a	3.33d	5.77c	14.20b	5.73b	4.33c	5.83b	11.20a
1.0	0	18.00b	14.37d	16.00c	18.93a	16.67d	26.20b	25.73c	32.17a
	30	14.57d	15.90c	17.40b	24.80a	15.50c	19.33b	17.67c	20.17a
	50	9.87d	21.83b	23.20a	13.37c	13.87c	21.77b	23.97a	21.83b
	60	22.43a	13.50d	14.87c	16.43b	7.87d	19.83b	20.40a	17.63c
	90	16.27c	22.50a	19.80b	15.80c	12.17bc	12.43b	11.87c	24.43a
	120	21.80a	14.90c	10.80d	20.67b	10.33b	5.77c	10.40b	19.23a
		LSD $_{(A\times B\times C)}$=0.50				LSD $_{(A\times B\times C)}$=0.444			

Root and Shoot Length (cm)

In Table 10, it was revealed that the root length decreased as the salt dose increased. The highest root length was obtained from May 505 genotype (30.93 cm) in pots with 60 mM NaCl dose and no salicylic acid application. In the experiment, the highest value Lazer genotype was observed after 0.5 mM salicylic acid application against salt stress. However, as the Laser genotype and salt dose increased, it was determined that salicylic acid applications were not effective on root length. By increasing the salicylic acid dose to 1.0 mM, the Lazer genotype with a root length of 22.43 cm at 60 mM NaCl stress and the May 505 genotype with a root length of 24.80 cm at 30 mM NaCl stress came to the fore. It was observed that the highest shoot length value was not salt stress, but May 505 genotype was obtained with 1.0 mM dose of salicylic acid. In terms of shoot length, May 505 genotype also gave high values under salt stress conditions. Salicylic acid doses (1.0 mM) under high salt stress to Lazer, Selin and May 455 genotypes did not have a positive effect on shoot length values. In pots without salt stress and salicylic acid applied, Selin genotype gave the highest shoot length of 28.50 cm. Increasing salt doses caused a significant decrease in shoot length in all cultivars with

salicylic acid. The Laser genotype gave a value of 21.83 cm with 0.5 mM SA at 30 mM salinity and the May 455 genotype gave a value of 21.23 cm. In black bean, salinity caused a decrease in seedling length, but priming with 10 mM salicylic acid gave the most effective result compared to 2 and 20 mM doses and the seedling length increased under salt stress (Heidarian & Roshandel, 2021). Hussein et al. (2007) emphasized that salicylic acid increased growth in maize against saline conditions. Salicylic acid increased growth and yield in chickpea under salt stress conditions (Riaz et al., 2019). Priming with salicylic acid suppressed the phytotoxic effects caused by salinity in cotton, affected plant growth, improved the phenotypic appearance of the plant and increased salt tolerance in cotton (Keya et al., 2023).

Root Fresh Weight and Shoot Fresh Weight (g)

In Table 11, the highest root fresh weight was observed in the Selin genotype (1.54 g), which was not treated with salicylic acid at 30 mM salt stress. It was revealed that root growth decreased in all cultivars in general at 120 mM NaCl dose, where salt stress was the highest. However, increasing the salicylic acid had a positive effect on root growth. The highest root fresh weight was obtained in the Selin genotype under control conditions. However, the root fresh weight of Selin genotype decreased significantly when salt doses were increased, except for 30 mM salt stress. In the applied 50 mM NaCl and 0.5 mM salicylic acid, the Laser genotype had the same value at 1.0 mM salicylic acid, and May 455 was in the front row with 0.88 g. The highest shoot fresh weight (90 mM salt + 0.5 mM salicylic acid) was obtained from May 455 genotype with 10.22 g (Table 11). In all other genotypes, shoot fresh weight decreased significantly as salt stress increased, regardless of salicylic acid dose. In the experiment where salicylic acid was not applied and the salt stress was 30 mM, the highest shoot fresh weight was obtained as 7.03 g from Selin genotype. As the salicylic acid dose increased in Selin genotype, shoot fresh weight decreased. Salt dosage caused a decrease in shoot fresh weight. When the salicylic acid dose was increased to 0.5 mM, the May 505 genotype ranked higher in high salt conditions. Further increasing the salicylic acid dose resulted in less decrease in fresh weight values. While high values were obtained at the 1.0 mM SA without salinity, the salicylic acid effect was less at the 120 mM salt dose. The highest value in shoot fresh weight values was obtained from applications without the use of salicylic acid. Salicylic acid-induced abiotic stress tolerance is due to the fact that osmolyte accumulation via salicylic acid helps maintain osmotic homeostasis and improves the regulation of mineral substance intake (Abdi et al., 2022). Salicylic acid to maize exposed to salt stress improved plant height, root and shoot fresh and dry weight (Khodary, 2004). Priming against salt stress caused an increase in seedling weight (Heidarian & Roshandel, 2021). Foliar salicylic acid application to cotton against salinity reduced the negative effect of salt on cotton seedlings (Hamani et al., 2021).

Root Dry Weight and Shoot Dry Weight (g)

The highest root dry weight was obtained from Selin genotype with 0.36 g (90 mM salt + 0 mM salicylic acid) (Table 12). However, in terms of root fresh weight, Selin genotype had the highest value at 30 mM salt stress. This shows that as the salt stress increases, the organic and inorganic substances in the roots decrease. Increasing the dose of salicylic acid also had no effect on root dry weight. Root dry weight values gave high values in cultivars that were not treated with salicylic acid under salt stress conditions.

Table 11. Effect of seed priming of salicylic acid on root and shoot fresh weight of cotton genotypes

SA	Salinity	Root fresh weight (g)				Shoot fresh weight (g)			
(mM)	(mM)	Lazer	Selin	May 455	May 505	Lazer	Selin	May 455	May 505
0	0	0.26d	0.74a	0.61b	0.37c	3.10b	6.81a	2.36d	2.53c
	30	0.75b	1.54a	0.43c	0.37d	3.91b	7.03a	2.90c	2.76d
	50	0.68a	0.07d	0.36c	0.41b	3.20a	0.35d	1.91c	2.61b
	60	0.36c	0.29d	0.67a	0.54b	2.61a	1.60c	2.23b	1.46d
	90	0.89b	1.50a	0.42d	0.50c	2.92a	2.83a	1.70c	2.09b
	120	0.09a	0.06b	0.06b	0.07b	0.35a	0.35a	0.35a	0.35a
0.5	0	0.35c	0.44b	0.50a	0.33d	2.11d	2.76c	3.48a	2.92b
	30	0.51b	0.74a	0.43c	0.28c	4.37a	2.85b	1.65d	2.08c
	50	0.88a	0.74b	0.53c	0.38d	3.32a	2.81c	2.98b	2.01d
	60	0.31b	0.31b	0.29c	0.46a	1.52c	1.68b	1.26d	1.87a
	90	0.09d	0.36b	0.21c	0.86a	0.61d	1.37c	10.22a	1.85b
	120	0.22a	0.06c	0.07b	0.23a	0.51b	0.35c	0.53b	0.96a
1.0	0	0.50c	0.46d	0.67a	0.57b	2.18d	4.98a	3.73c	4.77b
	30	0.35d	0.50c	0.64b	0.86a	2.34c	2.79b	2.25c	3.49a
	50	0.20d	0.84b	0.88a	0.36c	1.55c	2.90b	3.18a	3.09a
	60	0.23d	0.59a	0.53c	0.55b	0.63d	2.56b	2.76a	2.06c
	90	0.41b	0.32d	0.35c	0.57a	1.70b	1.33c	1.06d	2.96a
	120	0.40b	0.18d	0.20c	0.52a	0.82b	0.46c	0.89b	1.82a
		$LSD_{(A\times B\times C)}$=0.009				$LSD_{(A\times B\times C)}$=0.100			

Table 12 Effect of seed priming of salicylic acid on root and shoot dry weight of cotton genotypes

SA (mM)	Salinity (mM)	Root dry weight (g)				Shoot dry weight (g)			
		Lazer	Selin	May 455	May 505	Lazer	Selin	May 455	May 505
0	0	0.04c	0.09a	0.09a	0.05b	0.28b	0.74a	0.28b	0.28b
	30	0.10b	0.26a	0.08c	0.04d	0.44b	0.86a	0.33c	0.30d
	50	0.18a	0.01c	0.08b	0.03c	0.47a	0.08d	0.26c	0.37b
	60	0.06c	0.04c	0.28a	0.09b	0.30c	0.19d	0.44a	0.24b
	90	0.13b	0.36a	0.09c	0.08c	0.41b	0.48a	0.28d	0.32c
	120	0.02a	0.01a	0.01a	0.02a	0.05b	0.09a	0.08a	0.08a
0.5	0	0.04b	0.06b	0.09a	0.05b	0.27d	0.29c	0.48a	0.31b
	30	0.07b	0.16a	0.09b	0.04c	0.57a	0.34c	0.42b	0.23d
	50	0.15a	0.14a	0.11b	0.05c	0.52a	0.40b	0.39b	0.29c
	60	0.04a	0.05a	0.05a	0.05a	0.20b	0.21b	0.18c	0.27a
	90	0.02d	0.08b	0.04c	0.11a	0.09d	0.22c	0.23b	0.33a
	120	0.05a	0.01b	0.03ab	0.033a	0.09b	0.09b	0.09b	0.14a
1.0	0	0.07ab	0.05b	0.11a	0.09a	0.24d	0.59b	0.50c	0.63a
	30	0.09b	0.07b	0.09b	0.11a	0.36b	0.35b	0.36b	0.50a
	50	0.03d	0.17b	0.26a	0.06c	0.19d	0.43b	0.50a	0.42c
	60	0.08b	0.11a	0.10a	0.07b	0.17d	0.36b	0.42a	0.31c
	90	0.07b	0.06b	0.13a	0.07b	0.28b	0.20d	0.24c	0.49a
	120	0.04b	0.02c	0.02c	0.07a	0.21b	0.09d	0.19c	0.33a
		LSD $_{(A\times B\times C)}$=0.023				LSD $_{(A\times B\times C)}$=0.010			

Table 13 Effect of seed priming of salicylic acid on chlorophyll content index (SPAD) of cotton genotypes

SA (mM)	Salinity (mM)	Chlorophyll content index (SPAD)			
		Lazer	Selin	May 455	May 505
0	0	39.95c	46.92b	43.37bc	58.43a
	30	55.33b	44.67c	45.47c	61.13a
	50	53.87a	32.67c	46.80b	53.23a
	60	51.83bc	47.95c	60.98a	54.32b
	90	49.98b	57.00a	60.83a	58.48a
	120	33.66a	22.37b	31.73a	31.33a
0.5	0	42.39c	58.68a	48.18b	50.82b
	30	44.58b	40.85b	57.15a	59.32a
	50	55.67b	46.55c	40.75d	62.08a
	60	60.49a	41.93c	46.90b	60.67a
	90	48.60b	52.17b	52.16b	56.92a
	120	46.00b	32.37d	40.27c	60.10a
1.0	0	56.18b	60.20a	50.20c	45.20d
	30	49.80b	61.63a	58.72a	59.17a
	50	42.60c	47.12b	60.00a	43.12c
	60	61.28a	44.04b	61.27a	60.93a
	90	48.97b	50.83b	60.03a	59.32a
	120	61.55a	56.12b	49.23c	51.98c
		LSD $_{(A\times B\times C)}$= 3.959			

Selin was the genotype that gave the highest value in terms of shoot dry weight (Table 12). The highest value in terms of shoot dry weight was obtained from May 455 genotype. However, this value was found to be low in shoot dry weight. This situation can be interpreted as the high water intake capacity of May 455. In all cultivars, the increase in salt dose caused a decrease in shoot dry weight. However, May 505 genotype gave higher values at 1.0 mM salicylic acid dose compared to control conditions at 120 mM salt stress. This reveals that the May 505 genotype can be grown under high salt stress conditions with salicylic acid application. In the absence of salt stress, the Selin genotype may be preferred. Salicylic acid application to wheat increased root and shoot dry weight under salt stress conditions (Azeem et al., 2019; Abdi et al., 2022). Priming of baby corn seeds with SA in saline conditions (6 dS m^{-1}) significantly increased the root dry weight. At high salt concentrations (9-12 dS m^{-1}), treatment of seeds with salicylic acid had no significant effect. Priming baby corn seeds with 1 mM SA at moderate salinity has been suggested for production (Islam et al., 2022). Salicylic acid application under salt stress caused a significant increase in shoot dry weight in wheat (Arfan et al., 2005). Similar results were obtained in dry leaf weight of cotton (Hussein et al., 2012).

Chlorophyll Content Index (SPAD)

The highest SPAD value in plants was obtained from the May 505 genotype containing 0.5 mM salicylic acid under 50 mM salt stress (Table 13). In 0.5 mM salicylic acid application, SPAD value was higher than control at 120 mM salt stress in all cultivars. In applications where

salt stress is high, the addition of salicylic acid caused an increase in the SPAD values of the plants. The lowest SPAD value was observed in Selin genotype with 0.5 mM salicylic acid application at 120 mM salt stress.

Pancheva et al. (1996) emphasized that the application of more than 1 mM SA reduces the rate of photosynthesis. Soaking wheat seeds with salicylic acid against salinity significantly reduced NaCl-induced phytotoxicity in terms of chlorophyll index (Alam et al., 2022). SPAD values obtained after salicylic acid applied to *Vigna radiata* grown under salinity are an important criterion for evaluating plant lines, and it can be used as a physiological measurement criterion in determining photosynthetic performance in plants under salt stress (Ogunsiji et al., 2023). High NaCl content caused a decrease in chlorophyll content due to inhibition of chlorophyll biosynthesis in wheat (Khan, 2003b). Salicylic acid application in salt conditions improved chlorophyll value in cotton (Souza et al., 2023). Photosynthesis and chlorophyll synthesis are inhibited in plants exposed to high salt (Cha-Um et al., 2010; Mahboob et al., 2016; Mahboob et al., 2017), but salicylic acid reduces the negative effects (El Tayeb, 2005; Afzal et al., 2006; Farooq et al., 2007; Hussain et al., 2011; Rehman et al., 2011; Pirasteh-Anosheh et al., 2012). In salt stress conditions, SA to wheat improved seedling length and total chlorophyll content (Azeem et al., 2019). Salicylic acid application under salt stress conditions increased the chlorophyll content index (SPAD) in barley (Pirasteh-Anosheh et al., 2014). Similar studies were also observed in different plants (Khodary, 2004; Parida et al., 2008; Nikolaeva et al., 2010; Pirasteh-Anosheh & Emam, 2012). Hamid et al. (2010) revealed that priming wheat seeds with salicylic acid under salinity stress conditions made the seedlings stronger and increased the plant chlorophyll content.

Conclusion

In the present study, we explored the exogenous SA-induced salt tolerance in cotton. High dose salt concentrations (90, 120 mM) negatively affected germination and development in seeds. Salicylic acid application was significantly effective in the germination and seedling growth of cotton seeds. Therefore, to obtain high yields in cotton under saline conditions, priming the seeds with 0.5 mM SA will be effective. Priming with SA reduced the degradation of photosynthetic pigments while increasing plant biomass. Exogenously applied SA increased the salinity tolerance of Selin and May 505, particularly by reducing the negative effects of salts. Salicylic acid application (0.5 mM) had positive effects on germination percentage, plant height, fresh and dry weight and SPAD parameters in cotton. Selin and May 505 genotypes can be recommended to be planted after priming the seeds with salicylic acid (0.5 mM) for soils with 50 mM-60 mM salt concentration.

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The Effects of Different GA_3 and Mycorrhiza Dosages on Mini Tuber Production in Potatoes

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ABSTRACT

This study was conducted in 2017 under greenhouse conditions using selected four different potato clones to determine the effects of different doses of GA_3 and arbuscular mycorrhizal fungus on mini tuber production. The research, carried out in a randomized complete block design with three replications, applied GA_3 doses of 0, 5, 10, and 15 ppm, and mycorrhizal inoculat doses of 0, 500, 1250, and 2000 mg/100 tubers. Parameters including emergence time, plant height, main stem number, tuber number, average tuber weight, tuber size distribution (>45 mm, 28-45 mm, <28 mm), and maturity period were examined. The effect of GA3 application on all investigated parameters except the number of main stems was significant, statistically. The highest mini tuber number (9.1 tubers) and mini tuber yield (408.4 g/pot) were obtained from the application of 15 ppm GA_3, while the highest average mini tuber weight (46.74 g) was obtained from the control group. In mycorrhizal applications, the highest tuber number was obtained at a dose of 500 mg/100 tubers, and the highest mini tuber weight and yield were obtained at a dose of 1250 mg/100 tubers. As a result of the study, it was determined that the application of 15 ppm GA_3 is suitable due to its positive effect on mini tuber multiplication, and the mycorrhizal application at a dose of 500 mg/100 tubers is appropriate due to its positive effect on increase of tuber number.

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Introduction

Potato (*Solanum tuberosum* L.) holds significant importance worldwide as a staple food in terms of consumption and production quantity (Demirel et al., 2020). The first potato cultivation is reported to have taken place approximately 6000 years ago in the Andes mountains of America (Öztürk and Polat, 2017). In Europe, potato cultivation began in Ireland from the 18th century onwards (Laçin, 2018). Nowadays, potato cultivation is widespread in numerous countries (Vincent et al., 2013). The ability of potatoes to adapt well to different environments, provide high yields, possess processing characteristics, offer dietary diversity, and serve as a nutritious food source has contributed to its widespread cultivation from ancient times to the present. Besides being a rich source of carbohydrates and starch, potatoes are also abundant in various minerals and vitamins, including calcium. Potatoes are propagated through their tubers. Due to the importance of producing seed potatoes under controlled and sterile conditions to ensure disease-free and desired seed characteristics, controlled multiplication of basic seed potatoes is crucial (Jones, 1988; Struik and Lommen, 1990; Lommen, 1995). Meristem culture is utilized in potato seed production to obtain virus-free and healthier plants. Subsequently, mini tubers are obtained under controlled greenhouse conditions (Bryan, 1988; Yıldırım, 1995). The use of suitable environments for the multiplication of mini tubers under greenhouse conditions is essential. Apart from aerophonic or hydroponic methods, various substrates such as perlite, peat, sand, and different organic material mixtures are used as propagation media (Kaur et al., 2000). In potato seed production, it has been indicated that controlled conditions are more suitable for producing elite seed potatoes, which are the original seed class, in terms of tuber yield and multiplication compared to open-field conditions (Yılmaz et al., 2018). Gibberellins are plant growth regulators obtained from Gibberella fujikuroi fungi (Seçer, 1989). GA_3 is the most commonly used form of these in agriculture, known for its properties to promote seed germination and break dormancy (Olszewski et al., 2002; Tyler et al., 2004). Gibberellic acids are substances belonging to the class of hormones that promote plant growth and development (Aslantaş, 2012). Beneficial fungi are used in agricultural production for nutrition and plant protection purposes (Bhandari, 2021). Mycorrhizal fungi have two different types: endo- and ectomycorrhizae (Bonello, 2001). Endomycorrhizae

support plant growth in nutrient-poor soils. By forming a symbiotic relationship with plant roots, they improve the uptake of necessary nutrients from the soil. Through mutually beneficial biotic interactions, mycorrhizae obtain carbon from the plant while making the plant more efficient in using water and nutrients located far from the root zone (Mitra et al., 2020). Mycorrhizal fungi enhance plant resistance to drought, salinity, and heavy metal stress (Tisdall, 1994). Additionally, mycorrhizal fungi act as protective biological agents against pathogens by establishing a symbiotic life with the plant (Himaya, 2021). This study was conducted to determine the effects of different doses of GA_3 and Arbuscular Mycorrhizal fungi on potato development. The research was planned to propagate mini tubers obtained from meristem culture of the clones with good characteristics, namely 7/12, 3/110, 6/28, and 10/15, developed within the framework of the TÜBİTAK-TOVAG 214O115 project, and to obtain seed tubers necessary for different location trials to be established later. Clone number 6/28 used in the study was registered under the name GÜNGÖRBEY by the General Directorate of Seed Registration and Certification of the Ministry of Agriculture and Forestry of the Republic of Turkey on 06.04.2022, and it took its place in the National Variety List (Anonymous, 2024). This research was conducted in the polycarbonate greenhouses of the Department of Field Crops, Faculty of Agriculture, Gaziosmanpaşa University, in pot trials. The effects of different doses of mycorrhiza and gibberellic acid on the multiplication of mini tubers of selected clones were examined, and it was aimed to determine their effects on plant growth, tuber count, tuber size, tuber yield, and especially tuber multiplication rates in tubers planted in pots.

Materials and Methods

This study was conducted in 2017 following a randomized complete block design with three replications. The genotypes named 3/110, 6/28, 7/12 and 10/15 selected in our study were assigned to main plots, while different doses of GA_3 and mycorrhiza were assigned to subplots. Four different doses of GA_3 (0, 5, 10, and 15 ppm) and four different doses of mycorrhizal fungus (0, 500, 1250, and 2000 mg/100 tubers) were investigated. Trials containing GA_3 and mycorrhizal fungus were conducted separately in two different experimental setups. The mycorrhizal fungus Glamus spp. was used in the study. The mycorrhizal fungus used is commercially named shubhodayo and the microorganism name in its content is *Glomus proliferum*. The specified mycorrhiza doses were mixed with 375 ml of water, and 1 drop of spreader-sticker was added to the mixture. Mini tubers were immersed in the prepared solution for 1 minute and then immediately planted without exposure to sunlight. Observations and measurements conducted in this study, and the subsequent data acquisition and evaluation, were based on the methodologies outlined by Yılmaz (1993), Özkaynak and Samancı (2002), Karaat (2011), Yılmaz et al. (2014), and Karan and Yılmaz (2016). The obtained results were statistically analyzed using variance analysis according to the experimental design. The means of the results were compared using the Duncan multiple range test (Yurtsever, 1984).

Results and Discussion

Emergence Time (days)

The effect of different doses of GA_3 and mycorrhiza on emergence time is presented in Table 1. The emergence time ranged from 27.8 to 32.3 days with GA_3 application and from 26.56 to 29.83 days with mycorrhiza application. According to Table 1, while GA_3 application was statistically significant at the 1% level, mycorrhiza application did not create a statistically significant difference. The earliest emergence was achieved in the control group (0 ppm) with GA_3 application, followed by the second earliest emergence from the 15 ppm GA_3 dose. The 5 and 10 ppm GA_3 applications were statistically in the same group. In some cases, GA_3 application is known to cause secondary dormancy (Yılmaz, 2016). The average emergence time for the clones treated with GA_3 ranged from 19.0 to 40.5 days, with statistically significant differences observed. The clone with the earliest emergence was 6/28, followed by 10/15, 3/110 and 7/12 in order (Table 2). Similar to GA_3 applications, differences in emergence time were statistically significant in mycorrhiza applications as well. The average emergence time for clones ranged from 16 to 41.0 days. The clone with the earliest emergence was 6/28, followed by 10/15, 3/110, and 7/12 in order. In both GA_3 and mycorrhiza applications, the emergence time of clones in the control group varied, with clone 3/110 ranging from 30 to 35 days, clone 6/28 ranging from 16.25 to 19.0 days, clone 7/12 at 40.5 days, and clone 10/15 ranging from 26.25 to 27.5 days.

Table 1. The effect of GA3 and Mycorrhiza applications on plant yield parameters in mini tuber production of potato.

	ET	PH	MSC	TC	ATW	TY	Tuber Size Distribution			MP
							>45 mm	28-45 mm	<28mm >	
GA_3 Dosages										
0 ppm	27.8b	91.83a	1.40*	8.9a	46.74a	402.5a	34.49ab	27.34a	36.78b	122.8b
5 ppm	31.5a	68.64c	1.40	6.7b	39.23ab	275.8c	29.98b	17.00b	53.02a	124.5a
10 ppm	32.3a	76.00bc	1.10	8.7a	38.45b	361.5b	32.26b	16.89b	50.85a	124.5a
15 ppm	29.5ab	77.78b	1.40	9.1a	46.30ab	408.4a	42.50a	19.48ab	37.53b	124.5a
Mycorrhizal Dosages										
0 (mg/100 tuber)	26.56*	91.38b	1.4*	8.90bc	47.57a	402.0ab	36.74ab	26.49*	36.78*	122.8a
500 (mg/100 tuber)	29.83	99.11ab	1.4	11.10a	34.04b	369.1ab	31.36b	26.28	42.41	117.5b
1250 (mg/100 tuber)	27.17	97.86ab	1.2	8.40c	52.61a	403.3a	42.44a	19.18	38.38	117.5b
2000 (mg/100 tuber)	27.72	100.60a	1.2	10.10ab	39.01b	365.9b	39.10ab	27.07	33.83	117.5b

ET: Emergence Time (days); PH: Plant Height (cm); MSC: Main Stem Count (pieces); TC: Tuber Count (pieces); ATW: Average Tuber Weight (g); TY: Tuber Yield (g pot^{-1}); MP: Maturation Period (days), *: Difference between means is not significant, small letters show different groups at the 1 %

Table 2. Effect of GA3 doses (ppm) on yield parameters of potato clones

	3/110					6/28				
	0 ppm	5 ppm	10 ppm	15 ppm	Ort.	0 ppm	5 ppm	10 ppm	15 ppm	Ort.
ET	35bc	38ab	34bcd	30de	34B	16f	15f	27e	18.5f	19.0D
PH	83.11cde	43.11g	41.11g	57.22fg	56.14C	74.78def	65.44ef	65.44ef	77.00de	70.67B
MSC	1.11cd	1.00d	1.00d	1.30abcd	1.10B	2.00a	1.90ab	1.40abcd	1.80abc	1.80A
TC	6.10ghı	2.70j	4.30ıj	5.00hıj	4.5C	11.00bcd	6.30fghı	9.20cde	11.40abc	9.50AB
ATW	54.16bc	9.49g	21.37fg	40.53cde	31.39C	51.56bc	74.47a	54.83bc	52.09bc	58.24A
TY	328.3fg	25.5j	92.1j	201.2ı	161.8D	542.8bc	471.0cde	489.6cd	590.1ab	523.4A
MP	114d	114d	114d	114d	114D	121c	121c	121c	121c	121C
TSD	3/110					6/28				
	0	500	1250	2000	Ort.	0	500	1250	2000	Ort.
>45< mm	39.27 b-f	1.00g	4.18g	21.73defg	16.56D	48.13abc	44.44abc	50.20abc	58.72ab	50.73A
28-45 mm	33.79ab	3.70e	9.44de	23.35abcd	17.57A	11.50de	23.61abcd	19.37bcde	23.13abcd	19.40A
<28mm >	26.93de	95.30a	86.39a	54.93b	65.89A	40.37bcd	31.94cde	30.44cde	18.16e	30.23C

	7/12					10/15				
	0 ppm	5 ppm	10 ppm	15 ppm	Ort.	0 ppm	5 ppm	10 ppm	15 ppm	Ort.
ET	41.0a	42.0a	37.0ab	42.0a	40.5A	19f	31Cde	31cde	29de	27.5C
PH	66.00ef	99.00bc	106.20b	88.45bcd	89.92A	143.40a	67.00ef	91.22bcd	88.44bcd	97.53A
MSC	1.20bcd	1.70abcd	1.00d	1.20bcd	1.30B	1.20bcd	1.10cd	1.00d	1.44abcd	1.2B
TC	6.80fgh	8.70def	13.00a	7.20efgh	8.90B	12.00ab	9.20cde	8.20defg	12.60ab	10.50A
ATW	42.63bcde	52.95bc	49.05bcd	57.32b	50.49B	38.60cde	20.00fg	25.56ef	35.24def	30.60C
TY	287.9gh	423.1de	634.0a	405.4ef	437.6B	448.9de	183.7ı	230.2hı	436.7de	324.9C
MP	128.0 b	128.0 b	128.0 b	128.0 b	128.0 B	135a	135a	135a	135a	135A
TSD	7/12					10/15				
	0	500	1250	2000	Ort.	0	500	1250	2000	Ort.
>45< mm	19.74efg	55.85ab	42.61abcd	60.23a	40.61 B	39.82a-e	18.63fg	32.04cdef	29.33cdef	29.96C
28-45 mm	36.07a	16.63cde	20.92abcd	13.01cde	21.66A	27.99abc	24.05abcd	17.85bcde	19.89a-e	22.44A
<28mm >	47.61bcd	27.52de	36.48bcde	26.75de	34.59C	32.19cde	57.32b	50.11bc	50.28bc	47.47B

ET: Emergence Time (days); PH: Plant Height (cm); MSC: Main Stem Count (pieces); TC: Tuber Count (pieces); ATW: Average Tuber Weight (g); TY: Tuber Yield (g pot^{-1}); MP: Maturation Period (days); TSD: Tuber Size Distribution

Table 3. Effect of mycorrhizal doses (mg/100 tubers) on the yield parameters of clones

	3/110					6/28				
	0	500	1250	2000	Ort.	0	500	1250	2000	Ort.
ET	29.89cd	27.78d	25.67de	27.00d	27.58B	16.00f	15.00f	15.00f	19.00ef	16.25C
PH	83.11def	76.44defg	89.56cdef	83.78def	83.22B	47.78Efg	73.22fg	79.78defg	75.11defg	75.72B
MSC	1.11cd	1.00d	1.00d	1.30abcd	1.10B	2.00a	1.90ab	1.30abc	1.20bc	1.60A
TC	6.10ghı	7.40fgh	6.50gh	7.10gh	6.80C	10.60cde	10.20def	7.40fgh	9.10efg	9.30B
ATW	54.16bc	30.55fgh	46.51cde	33.00fgh	41.05B	51.56bcd	41.30c-g	60.55ab	40.45defg	48.50A
TY	328.3fg	220.10g	298.30efg	233.80fg	270.10B	542.80a	417.60bcd	436.00bc	396.60cde	441.5A
MP	114.0d	107.0e	107.0e	107.0e	108.8D	114.0d	114.0d	114.0d	114.0d	114.0C
TSD	3/110					6/28				
	0	500	1250	2000	Ort.	0	500	1250	2000	Ort.
>45< mm	39.27bcde	26.28cde	41.55a-e	39.11bcde	36.55AB	48.13abc	37.85bcde	62.67a	42.37abcd	47.75A
28-45 mm	33.80a	27.12ab	24.28ab	27.71ab	28.23A	11.50bc	17.24ab	17.54ab	27.29ab	18.39A
<28mm >	26.93b	46.60ab	34.17ab	33.18ab	35.27A	40.37ab	44.91ab	19.79b	30.34ab	33.85A

	7/12					10/15				
	0	500	1250	2000	Ort.	0	500	1250	2000	Ort.
ET	41.00ab	48.00a	39.44b	36.33bc	40.5A	19.33ef	28.55d	28.55d	28.55d	26.25B
PH	66.00g	101.60c	91.89cd	91.33cde	87.69B	143.40ab	145.20ab	130.20b	152.30a	142.80A
MSC	1.20bc	1.00c	1.10c	1.20bc	1.10B	1.20bc	1.30abc	1.20bc	1.20bc	1.20AB
TC	6.80gh	12.10bcd	6.50gh	6.90gh	8.10BC	12.00bcde	14.70ab	13.40bc	17.30a	14.40A
ATW	42.63cdef	36.82efgh	72.36a	54.11bc	51.48A	41.93cdef	27.51h	31.02fgh	28.35gh	32.50C
TY	287.90efg	436.00bc	468.30abc	375.10cde	391.80A	448.90abc	402.70bcd	410.50bcd	485.30ab	436.90A
MP	128.0b	121.0c	121.0c	121.0c	122.8B	135.0a	128.0b	128.0b	128.0b	129.8A
TSD	7/12					10/15				
	0	500	1250	2000	Ort.	0	500	1250	2000	Ort.
>45< mm	19.74e	30.03bcde	42.67abcd	49.63ab	35.52AB	39.82bcde	31.10bcde	22.87de	25.30de	29.82AB
28-45 mm	32.65a	32.14a	16.07ab	24.63ab	26.37A	27.99ab	28.61ab	18.83ab	28.65ab	26.02A
<28mm >	47.61ab	37.83ab	41.26ab	25.74b	38.11A	32.19ab	40.09ab	58.30a	46.05ab	44.16A

ET: Emergence Time (days); PH: Plant Height (cm); MSC: Main Stem Count (pieces); TC: Tuber Count (pieces); ATW: Average Tuber Weight (g); TY: Tuber Yield (g pot^{-1}); MP: Maturation Period (days); TSD: Tuber Size Distribution

Plant Height (cm)

Statistically significant differences at the 1% level were found in the plant heights of potato clones under both GA_3 and mycorrhiza applications. The reason for the higher plant height in the control group is thought to be due to the earlier emergence of plants in the control group (Table 1). In mycorrhiza applications, plant height ranged from 91.38 to 100.60 cm. The highest plant height was obtained from the application of 2000 mg/100 tubers of mycorrhiza, while the lowest plant height was observed in the control group. Mycorrhiza applications at doses of 500 and 1250 mg/100 tubers were statistically in the same group. Statistically significant differences were observed in the average plant heights of clones treated with GA_3. Plant height ranged from 56.14 to 97.53 cm. The highest plant height was recorded in clone 10/15 at 97.53 cm, followed by clones 7/12, 6/28, and 3/110. Clone 7/12 and clone 10/15 were statistically in the same group (Table 2). In mycorrhiza-treated clones, plant height ranged from 75.72 to 142.80 cm. The highest plant height was observed in clone 10/15, with other clones statistically in the same group. Plant height in the control group varied, with clone 3/110 at 83.11 cm, clone 6/28 at 74.78 cm, clone 7/12 at 66.0 cm, and clone 10/15 at 143.40 cm. In a study conducted by Atasever (2019) under field conditions, plant heights were determined as 76.8 cm in clone number 3/110, 79.6 cm in clone number 6/28, 78.1 cm in clone number 7/12 and 121.6 cm in clone number 10/15.

Main Stem Number (pieces plant^{-1})

The applied doses of GA_3 and mycorrhiza did not create statistically significant differences in the main stem number per plant. The main stem number ranged from 1.10 to 1.40 stems per plant with GA_3 application and from 1.2 to 1.4 stems per plant with mycorrhiza application. The reason for the lack of significant change in the main stem count due to GA_3 and mycorrhiza applications is that main stems emerge from the eyes on the seed tuber and GA_3 and mycorrhiza applications do not affect eye formation. The main stem counts of clones treated with GA_3 are presented in Table 2. The average stem count of clones was statistically significant, with the highest stem count of 1.80 stems obtained from clone 6/28, while other clones were statistically in the same group. Similarly, statistically significant differences were found in the main stem counts of clones treated with mycorrhiza, with the highest stem count obtained from clone 6/28. Other clones were statistically in the same group (Table 3). When examining the main stem counts of plants in the control group, counts ranged from 1.11 to 2.0 stems per plant. Clone 6/28 had the highest main stem counts among the control group. In the study by Atasever (2019), the number of main stems of clones numbered 3/110, 6/28, 7/12 and 10/15 was determined as 1.8, 2.0, 1.3 and 2.0, respectively.

Tuber Number (pieces plant^{-1})

The effect of different doses of GA_3 and mycorrhiza on tuber count was found to be statistically significant at the 1% level. The tuber count ranged from 6.7 to 9.1 tubers in GA_3 applications. The highest tuber count was obtained from the application of 15 ppm GA3, while the lowest count was from the 5 ppm GA_3 dose. In mycorrhiza applications, the highest tuber count was obtained from the 500 mg/100 tuber dose. The tuber count per plant ranged from 8.40 to 11.10 in mycorrhiza applications. This positive change is thought to be due to the better development of roots and below-ground parts with mycorrhiza application. Statistically significant differences were found in tuber counts among clones in GA_3 applications. The highest tuber count was obtained from clone 10/15 with 10.50 tubers per pot, followed by clones 6/28, 7/12, and 3/110. The average tuber counts of clones treated with mycorrhiza are presented in Table 3. Differences in tuber counts were statistically significant, showing similarities with tuber counts in GA_3 applications. From the control group, the highest tuber count per pot was obtained from clone 10/15, followed by clones 6/28, 7/12, and 3/110. In a study conducted by Öztürk (2022), tuber counts ranged from 5.8 to 10.3 tubers.

Average Tuber Weight (g)

The effect of different doses of GA_3 and mycorrhiza on average tuber weight is presented in Table 1. The impact of GA_3 and mycorrhiza applications on average tuber weight was found to be statistically significant at the 1% level. In GA_3 applications, the average tuber weight varied between 38.45 and 46.74 g. The negative effect of GA_3 application on average tuber weight is interpreted as being due to better vegetative growth of plants, resulting in the formation of numerous tubers with insufficient enlargement. Similar results regarding average tuber weight have been reported in studies by Haverkort and Marinus (1995), Mattar and Abdul (1988), Struick et al. (1989), and Mikitzel (1993). In mycorrhiza applications, the average mini-tuber weight ranged from 34.04 to 52.61 g. The highest average tuber weight was obtained from the application of 1250 mg/100 tubers of mycorrhiza, indicating that mycorrhiza applications increase mini-tuber size. Among GA_3-treated clones, clone 6/28 exhibited the highest average tuber weight. Statistically significant differences were observed among clones in terms of average tuber weight (Table 2). In mycorrhiza-treated clones, clone 7/12 had the highest average tuber weight and was statistically in the same group as clone 6/28. When comparing average tuber weights of untreated clones, clone 3/110 had the highest weight, followed by clones 6/28, 7/12, and 10/15.

Tuber Yield (g pot^{-1})

The effect of different GA_3 and mycorrhiza doses on mini-tuber yield was found to be statistically significant at the 1% level. Tuber yield averages ranged from 275.8 to 408.4 g/pot in GA_3 doses. The highest mini-tuber yield was obtained from the application of 15 ppm GA_3, which was statistically in the same group as the control group. In other studies, Abdala et al. (2000) mentioned that an increase in the number and length of stolons in potatoes leads to an increase in tuber count but may hinder tuber enlargement. For mycorrhiza applications, the highest tuber yield was obtained from the 1250 mg/100 tubers dose, while the lowest yield was obtained from the 2000 mg/100 tubers dose. Statistically significant differences were observed among clones in terms of tuber yield. Clone 6/28 had the highest tuber yield in both treatments. Among control groups, clone 6/28 had a higher tuber yield compared to other clones.

Tuber Size Distribution (%)

Values obtained for tuber size distribution were found to be statistically significant at the 1% level in both GA_3 and mycorrhiza applications. In GA_3 applications, the highest values were obtained from the >45 mm tuber size category in the 15 ppm GA_3 application, the 28-45 mm category in the control group, and the <28 mm category in the 5 ppm GA3 application. Both the 5 ppm and 10 ppm GA_3 applications were statistically in the same group for all three tuber size distributions. In mycorrhiza applications, statistically significant differences were observed only in the >45 mm tuber size category. Among GA_3 applications, there was no statistically significant difference in tuber size distribution between clones in the 28-45 mm category, while clone 6/28 had the lowest number of tubers smaller than 28 mm and the highest number of tubers larger than 45 mm. When examining the effect of mycorrhiza applications on tuber size distribution, clone 6/28 exhibited similarities with GA3 applications (Table 2). In another study aimed at seed potato tuber production by Şanlı and Cirit (2020), pre-planting application of 0, 1.5, 3.0, and 4.5 ppm GA_3 to seed tubers resulted in a statistically significant increase in tubers between 25-35 mm, while doses other than 1.5 ppm GA_3 significantly reduced the number of tubers larger than 60 mm.

Maturation Period (days)

GA_3 applications have been found to extend the maturation period, but the applied doses did not show statistical differences. The maturation period in the control group was determined to be 122.8 days, while it was 124.5 days in the GA_3-treated plants. On the other hand, mycorrhiza applications have been observed to shorten the maturation period compared to the control group, but there was no statistical difference among the applied mycorrhiza doses. The maturation period in the control group was determined to be 122.8 days, whereas it was 117.5 days in mycorrhiza-treated plants. When examining clones in terms of maturation period, according to Tables 2 and 3, it was determined that the clone with the earliest maturation period in both applications was 3/110, followed by clones 6/28, 7/12, and 10/15, respectively. Significant differences were found statistically among clone maturation periods in both applications. In the study conducted by Atasever (2019), the maturation period of the clones were determined as 119 days for clone number 3/110, 123 days for clone number 6/28, 119 days for clone number 7/12, and 113 days for clone number 10/15.

Conclusion and Recommendation

According to the results obtained, GA_3 and mycorrhiza applications did not have a positive effect on emergence time, did not cause a statistical change in the number of main stems in plants, while GA_3 application had a shortening effect on plant height, and mycorrhiza applications had an increasing effect. When the yield parameters were examined, it was determined that GA_3 application decreased the number, average weight, and yield of mini tubers per plant. However, mycorrhiza application increased the number of mini tubers but did not have a positive effect on the average weight and yield of mini tubers. For mini tuber propagation, mycorrhiza application at a dose of 500 mg/100 tubers is recommended.

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Prediction of Live Weight and Carcass Characteristics from Linear Body Measurements of Yearling Male Local Sheep

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ABSTRACT

Measurements of the body structure in sheep are worthy of judging the quantitative features of meat and useful in developing appropriate selection requirements. The current study was aimed to predict live weight and hot carcass weight from linear body measurements of yearling male local sheep. 84 days feeding period fortnightly taken data on 24 local sheep for body weight, body length, heart girth, wither height, sub-sternal height, tail length, tail width, scrotal circumference, and scrotal length were analyzed to study the relationship between linear body measurements and body weight. At the end of the trial all sheep were slaughtered to measure the relationship between body measurements, and hot carcass weight. Microsoft Excel 2010 was used for data analysis. The relationships between the various body measurements were calculated using pearson's correlation coefficient. The backward stepwise multiple regression procedure was used for the determination of the most suitable model for the prediction of the live weight and hot carcass weight. Hot carcass weight was highly correlated ($P<0.01$) with body weight and scrotal circumference. Besides, it was significantly ($P<0.05$) correlated with tail width. Body weight was significantly ($P<0.05$) correlated with all body measurements except tail length and scrotal length. It is concluded that the body weight of the local sheep can be predicted with heart girth, sub-sternal height and tail width; the equation is LW= -97.2 + 0.36HG + 2.1SBSH + 0.57TW with a better coefficient of determination; R^2 = 0.55 and the hot carcass weight can be predicted with sub-sternal height and tail width; the equation is HCW= -75.66 + 1.75SBSH + 0.85TW with a coefficient of determination; R^2 = 0.33. But, hot carcass can be predicted with body weight, the equation is HCW= -9.39+0.85BWT when weighing scales are affordable with a better coefficient of determination; R^2= 0.557.

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Introduction

Sheep meat production is vital to meet the protein needs of the consumers all over the globe. Most scientific studies concerning growth, one of the critical characteristics of sheep production have been conducted to raise meat production per sheep. Monitoring the development of sheep and estimation of genetic correlation between body weight and body measurements require evidence of body weight with related body measurements (Mohammad et al., 2012).

Body weight is a paramount trait in meat animals due to its direct implication for profit (Cam et al., 2010). Body measurements are significant in terms of manifesting the breed information (Riva et al., 2004; Verma et al., 2019) and necessary in providing data about the anatomy and physical performance of the breed. Measurements of various morphological structures are valuable in determining the quantitative traits of meat and are also essential in establishing suitable selection criteria (Sharaby & Suleiman, 2013; Islam et al., 1991).

The physical performance of animals can be showed by linear body measurements (Goe et al., 2001; Attah et al., 2004) and are also important in estimating body weight and carcass traits (Atta & El Khidir, 2004; Thiruvenkadan, 2005). Besides, body weight estimation is required for deciding the appropriate medicinal prescription, feed amount, and selling of the animals (Eyduran et al., 2013).

Body weight is rarely measured by the owners in rural areas due to inaccessibility of weighing scales and difficulty of weighing in field conditions, even though it is a requisite in economic feasibility decisions. As a result, marketing of animals is mostly carried out by negotiation based on their physical look, visual judgment, and loin-eye-area palpation which is biased (Grum et al., 2012) and technically imprecise (Otoikhian et al., 2008).

Many research works have been reported on the prediction of body weight and carcass characteristics based on linear body measurements. However, breed, gender, birth type, dam age at lambing, and management system affect body weight (Yilmaz et al., 2012).

Hence the present study is carried out to establish the relationship between live body weight and hot carcass

weight with some linear body measurements in local sheep as a step towards establishing a prediction equation to estimate the live body weight and hot carcass weight of sheep under field conditions without using a weighing scale.

Materials and Methods

Description of the Study Area

The study was conducted at Habru district Sirinka Agricultural Research Center, breed evaluation and distribution site in the eastern Amhara region of Ethiopia. The site is located about 508 km northeast of Addis Ababa at the geographical location between 11°45'0.42"N latitude and 39°36'52.21"E longitude. The map of the study area was presented in Figure 2.

The center is situated at an elevation of 1850 meters above sea level; a bi-modal type of rainfall receiving a mean annual rainfall of about 950 mm. Habru is one of the thirteen districts in the North Wollo zone. It is situated at an altitude ranging from 1200-2350 m.a.s.l at 11°35'N latitude and 39° 38'E longitude. Its mean annual maximum and minimum temperatures were 28.5 °C and 15 °C, respectively. Whereas, the mean annual rainfall of the district varied from 750 to 1000 mm (Mohammed et al., 2014).

Ethical Approval Certificate

This experiment was approved by Amhara Agricultural Research Institute research review forum and decision was obtained from Sirinka Agricultural Research Center for the study using live animals and before slaughtered sheep was used for the experiment with decision number 495/0020/2024 and date 22/01/2024.

Data Collection Methods

Twenty-four yearling intact local sheep with a mean initial body weight of 23.9 ± 1.9kg were purchased from a local market and housed in individual pens with raised slatted floors.

The animals were examined for their linear body measurements and the exact points at which the body measurements were taken [body length (F-G); height at withers (A-B); heart girth (C-D); sub-sternal height (D-E); tail length (J-K); tail width (L-M); scrotal length (H-I); scrotum circumference (N-O)] showed in Figure 1.

The experimental animals were grouped into six blocks with four male lambs in each block based on the initial body weight. Body weights and other body measurements of the animals were taken at the beginning of the trial and every fortnight during the 84 days of the feeding period. All animals were weighed in the morning hours after overnight fasting using a suspended weighing scale with a 50 kg capacity of 200 g precision and other body measurements were taken using a plastic measuring tape. At the end of the experiment all sheep were slaughtered to measure hot carcass weight. Linear body measurements that were measured and their description are mentioned in Table 1.

Data Analysis

Microsoft Excel 2010 was used to analyses the average of fortnight body measurements taken during 84 days of the feeding period. The relationships between the various body measurements were calculated using pearson's correlation coefficient. The backward stepwise multiple regression procedure was used for the determination of the most suitable model in the prediction of the live weight and hot carcass weight using various body measurements and this enabled to establish regression equations. In the analysis process, both body weight and hot carcass weight were considered as dependent variables. In addition, body weight was considered as independent variable to predict hot carcass weight in the presence of weighing scales or following live weight estimation.

The model for the analysis of multiple linear regressions was:

$Yi = Bo + \beta1x1 + \beta2x2 + \beta3x3 + \beta4x4 + \beta5x5 + \beta6x6 + \beta7x7 + \beta8x8 + ei$

Where:

Yi = the response variable; body weight and hot carcass weight

Bo = the intercept

x1, x2, x3,..., and x8 are the explanatory variables body length, heart girth, wither height, sub-sternal height, tail length, tail width, scrotal circumference and scrotal length, respectively

β1, β2, β3.. and β8 are regression coefficient of the variables x1, x2, x3... x8

ei = the residual random error.

NB: Body weight will be explanatory variable to predict hot carcass weight in the presence of weighing scale or following live weight estimation. But, it will be dependent variable in the absence of weighing scales. As a result, the multiple regression model will be adjusted in accordance with the predictive explanatory factors.

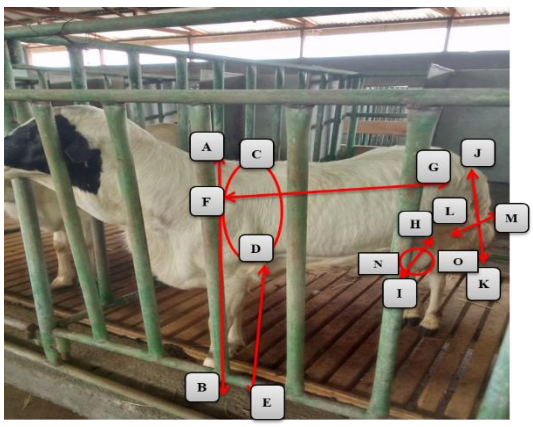

Figure 1. Displaying the exact locations of the body measures of yearling local male sheep

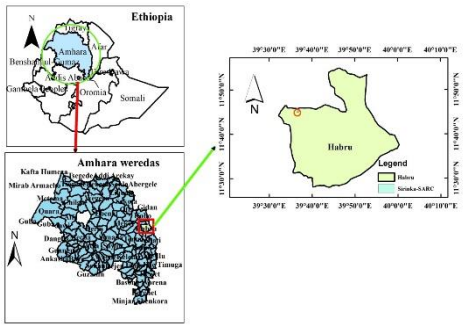

Figure 2. The Map of Study Area

Table 1. Linear body measurements measured in the experiments

Parameters	Descriptions
Body length (F-G)	Distance from the anterior shoulder point to the posterior extremity of the pin bone (cm)
Height at withers (A-B)	Vertical distance from the highest point of the shoulder (withers) to the ground surface at the level of the forelegs (cm)
Heart girth (C-D)	The body circumference at a point immediately posterior to the front leg and shoulder and perpendicular to the body axis (cm)
Sub-sternal height (D-E)	The height from the ground to the underside of the front body (cm)
Tail length (J-K)	Distance from the base to the tip of tail (cm).
Tail width (L-M)	Diameter at the midst of tail (cm)
Scrotal length (H-I)	Distance from the base to the tip of scrotum (cm)
Scrotal circumference (N-O)	Circumference at midst point of scrotum (cm)

cm: centimeter

Table 2. Pearson correlation coefficients between hot carcass weight and other linear body measurements

Variables	HCW	BWT	BL	HG	WH	SBSH	TL	TW	SC	SL
HCW										
BWT	0.759**									
BL	0.367ns	0.447*								
HG	0.285ns	0.430*	0.196ns							
WH	0.388ns	0.515*	0.742**	0.273ns						
SBSH	0.306ns	0.412*	0.457*	-0.249ns	0.601**					
TL	0.063ns	0.130ns	0.080ns	0.140ns	0.058ns	-0.137ns				
TW	0.429*	0.409*	0.025ns	0.440*	0.145ns	-0.303ns	0.397ns			
SC	0.547**	0.459*	0.208ns	-0.055ns	0.420*	0.420*	0.217ns	0.242ns		
SL	0.379ns	0.199ns	0.331ns	-0.040ns	0.535**	0.437*	0.143ns	0.096ns	0.507*	

ns= non significant, *= significant, **= highly significant, HCW= hot carcass weight, BW= body weight, BL= body length, HG= heart girth, WH= wither height, SBSH=sub-sternal height; TL= tail length, TW= tail width, SC= scrotal circumference, SL=scrotal length

Table 3. Prediction of body weight from linear body measurements in yearling male local sheep

Eq. No	Prediction equations	Adj. R^2
1	BW= -99.84 + 0.27BL + 0.4HG - 0.26WH + 2.11SBSH - 0.09TL + 0.54TW + 0.34SC - 0.38SL	0.52
2	BW= -101.85 + 0.26BL + 0.4HG - 0.25WH + 2.13SBSH + 0.52TW + 0.33SC - 0.39SL	0.55
3	BW= -92.7 + 0.14BL + 0.35HG + 1.82SBSH + 0.49TW + 0.31SC - 0.49SL	0.56
4	BW= -96.76 + 0.38HG + 2.04SBSH + 0.5TW + 0.3SC - 0.45SL	0.57
5	BW= -90.54 + 0.38HG + 1.81SBSH + 0.47TW + 0.21SC	0.55
6	BW= -97.2 + 0.36HG + 2.1SBSH + 0.57TW	0.55

BW= body weight, BL= body length, HG= heart girth, WH= wither height, SBSH=sub-sternal height; TL= tail length, TW= tail width, SC= scrotal circumference, SL=scrotal length and R^2= coefficient of determination

Result and Discussion

Body Measurements, Body Weight and Hot Carcass Weight Relationships

The pearson correlation coefficients between hot carcass weight and other linear body measurements are described in Table 2.

Hot carcass weight was highly correlated ($P<0.01$) with body weight and scrotal circumference. Besides, it was significantly ($P<0.05$) correlated with tail width. Body weight was significantly ($P<0.05$) correlated with all body measurements except tail length and scrotal length. The high correlation would imply measurements can be used as an indirect selection trait to advance body weight or could be used to estimate body weight (Ra et al., 2018), (Fasae et al., 2005) and (Gebremichael, 2008). The high correlation coefficients between live weight and body measurements suggest that either of these variables or their combination could provide a good prediction for estimating body weight of local sheep.

Prediction of Body Weight and Hot Carcass Weight from Linear Body Measurements

Prediction of Body Weight from Linear Body Measurements

Several regression equations were constructed using a backward stepwise regression procedure for the prediction of live weight from linear body measurements in Table 3.

When all the body measurements were included in the prediction equation the accuracy of the prediction was 0.52 (Eq. No.1). The results indicated that as all linear body measurements were included in the prediction equation a lesser coefficient of determination (R^2) was obtained. However, the body weight of the sheep can be predicted with only HG, SBSH and TW; the equation is LW= -97.2 + 0.36HG + 2.1SBSH + 0.57TW with better coefficient of determination, $R^2 = 0.55$. The same accuracy of prediction was obtained by incorporating seven and four traits (Eq. No. 2 and 5).

Table 4. Prediction of hot carcass weight from linear body measurements in Yearling male Local sheep (in absence of weighing scales)

Eq. No	Prediction equations	Adj. R^2
1	HCW= -78.78 + 0.46BL + 0.31HG -0.6WH + 1.43SBSH - 0.48TL + 0.67TW + 0.55SC + 0.37SL	0.39
2	HCW= -80.05 + 0.44BL + 0.29HG - 0.5WH + 1.46SBSH - 0.45TL + 0.67TW + 0.6SC	0.41
3	HCW= -90.16 + 0.4BL + 0.29HG - 0.48WH + 1.57SBSH + 0.57TW + 0.56SC	0.40
4	HCW= -70.45 + 0.16BL + 0.19HG + 0.9SBSH + 0.49TW + 0.48SC	0.38
5	HCW= -75.78 + 0.23HG + 1.18SBSH + 0.5TW + 0.48SC	0.39
6	HCW= -58.6 + 1.14SBSH + 0.66TW + 0.43SC	0.38
7	HCW= -75.66 + 1.75SBSH + 0.85TW	0.33

HCW= hot carcass weight, BL= body length, HG= heart girth, WH= wither height, SBSH=sub-sternal height; TL= tail length, TW= tail width, SC= scrotal circumference, SL=scrotal length and R^2= coefficient of determination

Table 5. Prediction of hot carcass weight from linear body measurements after live weight estimation or when weighing scale is available

Eq. No	Prediction equations	Adj. R^2
1	HCW=-10.93+0.68BWT+0.28BL+0.04HG-0.43WH+0.003SBSH-0.42TL+0.3TW+0.32SC+0.63SL	0.541
2	HCW=-10.81+0.68BWT+0.28BL+0.04HG-0.43WH-0.42TL+0.3TW+0.3SC+0.63SL	0.571
3	HCW=-8.86+0.7BWT+0.28BL-0.41WH-0.42TL+0.32TW+0.3SC+0.62SL	0.597
4	HCW=-5.84+0.75BWT-0.22WH-0.36TL+0.26TW+0.25SC+0.61SL	0.592
5	HCW=-4.65+0.82BWT-0.22WH-0.32TL+0.27TW+0.79SL	0.591
6	HCW=-9.72+0.82BWT-0.21WH+0.18TW+0.74SL	0.592
7	HCW=-6.19+0.89BWT-0.23WH+0.76SL	0.598
8	HCW=-15.51+0.79BWT+0.56SL	0.595
9	HCW=-9.39+0.85BWT	0.557

HCW= hot carcass weight, BWT= body weight, BL= body length, HG= heart girth, WH= wither height, SBSH=sub-sternal height; TL= tail length, TW= tail width, SC= scrotal circumference, SL=scrotal length and R^2= coefficient of determination

It was observed that the heart girth, sub-sternal height, and tail width were useful and trustworthy traits in body weight prediction for sheep. Heart girth as an important indicator of live weight was also reported by Atta & El Khidir (2004) in Nilotic sheep, Kumar et al. (2017) in Harnali sheep, Cam et al. (2010) in Karayaka, Tadesse and Gebremariam (2010) in Highland, Musa et al. (2012) in Sudanese Shogun , and Ravimurugan et al. (2013) in Kilakarsal sheep.

The estimation of live body weight based on heart girth augmented the coefficient of determination similar to that reported by Atta & El Khidir (2004), Johanson & Hildman (1954), and El-Khidir (1980). Lawrence & Fowler (1997) observed that skeletal measurements (wither height and body length) were less variable to estimate body weight compared to heart girth in agreement with this study.

Prediction of Hot Carcass Weight from Linear Body Measurements

Several regression equations were constructed using a backward stepwise regression procedure for the prediction of hot carcass weight from linear body measurements in Table 4.

When all the body measurements were included in the prediction equation the accuracy of prediction was 0.39 (Eq. No.1). The results indicated that as all linear body measurements were included in the prediction equation a better coefficient of determination (R^2) was obtained. However, the hot carcass weight of the local sheep can be predicted with only SBSH and TW; the equation is HCW= -75.66 + 1.75SBSH + 0.85TW with the coefficient of determination; R^2 = 0.33. The better accuracy of the prediction was obtained by incorporating other body measurements in addition to sub-sternal height and tail width.

In addition, alternative regression equations were constructed to predict hot carcass weight when weighing scales are available or following live weight estimation (Table 5). The results indicated that as all linear body measurements were included in the prediction equation a lesser coefficient of determination; R^2= 0.541 was obtained. Therefore, the alternative hot carcass weight prediction model can be with body weight; the equation is HCW= -9.39 + 0.85BWT with a better coefficient of determination; R^2 = 0.557. The better accuracy of the prediction was obtained by incorporating other body measurements in addition to body weight.

Table 6 shows the differences obtained between measured and estimated live weight and hot carcass weight (kg) with prediction equations of LW= -97.2 + 0.36HG + 2.1SBSH + 0.57TW; HCW= -75.66 + 1.75SBSH + 0.85TW and/or HCW= -9.39 + 0.85BWT. This result indicates that a maximum of 3kg difference from measured and predicted live weight and a maximum of 3.2kg difference from actual and predicted hot carcass weight were obtained in the absence of weighing scales. Whereas, a maximum of 3.6kg difference from actual and predicted hot carcass weight was obtained in the presence of weighing scales.

Table 6. The actual and predicted live weight and hot carcass weight of experimental animals

S.No	MLW	PLW	D	MHCW	PHCW-1	D	PHCW-2	D
1	26.5	26.9	0.4	11.9	12.6	0.7	13.1	1.2
2	27.9	26.7	1.2	12.9	14.1	1.2	14.3	1.4
3	23.7	26.7	3.0	10.8	13.9	3.1	10.8	0.0
4	24.9	25.0	0.1	12.0	12.0	0.0	11.8	0.2
5	25.7	26.0	0.4	11.2	12.4	1.2	12.4	1.2
6	30.3	28.0	2.3	14.6	14.1	0.5	16.3	1.7
7	26.2	25.8	0.3	12.1	13.0	0.9	12.8	0.7
8	27.1	27.5	0.4	11.2	13.3	2.1	13.7	2.5
9	27.7	28.1	0.4	13.8	15.4	1.6	14.2	0.4
10	25.7	24.3	1.4	12.0	11.0	1.0	12.4	0.4
11	28.0	27.2	0.8	13.0	13.9	0.9	14.4	1.4
12	25.2	25.5	0.4	12.0	12.9	0.9	12.0	0.0
13	25.0	27.0	2.0	12.2	13.5	1.3	11.8	0.4
14	26.8	27.0	0.2	12.8	14.7	1.9	13.4	0.6
15	27.7	26.2	1.5	14.0	12.7	1.3	14.2	0.2
16	27.4	28.3	0.8	13.6	13.3	0.3	13.9	0.3
17	25.0	25.8	0.8	11.2	12.8	1.6	11.9	0.7
18	28.6	30.2	1.6	13.6	16.3	2.7	14.9	1.3
19	28.6	27.2	1.4	15.8	13.7	2.1	14.9	0.9
20	23.8	24.8	1.0	14.4	12.1	2.3	10.8	3.6
21	28.0	27.7	0.4	16.0	15.0	1.0	14.4	1.6
22	27.0	27.7	0.7	13.8	12.8	1.0	13.5	0.3
23	30.1	29.8	0.3	18.8	16.3	2.5	16.2	2.6
24	31.1	29.9	1.3	19.0	15.8	3.2	17.0	2.0

HCW= Hot carcass weight; MLW: Measured live weight; PLW: Predicted live weight; D: Difference; MHCW: Measured HCW; PHCW-1: Predicted HCW (absence weighing scales); PHCW-2: Predicted HCW (presence weighing scales)

Conclusion

It is concluded that live weight of sheep can be predicted with heart girth, sub-sternal height and tail width under field conditions. Hot carcass can be predicted with the sub-sternal height and tail width of the animals in the absence of weighing scales. But, hot carcass weight can be predicted with body weight when weighing scales are affordable and after live weight estimation using prediction equations. This prediction method could be used for various purposes such as record keeping, estimating sheep's economic value, selection and genetic resource conservation. This result demonstrates that the same research efforts need to be undertaken with large sample size and incorporating other important morphological traits.

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Conflict of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

Data Availability

Data used to support the findings of this study are available from the corresponding author upon request.

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Response of spring rice (*Oryza sativa* L.) varieties to different nitrogen application methods at Nawalparasi West, Nepal

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ABSTRACT

Rice (*Oryza sativa* L.) cultivation necessitates an adequate supply of nitrogen to achieve optimal growth and yield. This study, conducted in Nawalparasi West from February to June 2023, aimed to assess the effects of nitrogen management through a foliar spray of nano urea, compared to need-based nitrogen management using the Leaf Color Chart (LCC) and the Recommended Dose of urea Fertilizer (RDF) application. The experiment followed a Randomized Complete Block Design (RCBD) with three replications, incorporating four nitrogen management levels (Control, RDF through urea fertilizer (120 kg/ha), 25% of RDF through basal urea (30 kg/ha) + nano urea, and 25% of RDF through basal urea (30 kg/ha) + (LCC) and two rice varieties (Chaite-5 and Hardinath-1). The results indicated that the LCC-based treatment produced the highest grain yield at 5.18 mt/ha, statistically similar to the yield of the nano urea-based treatment (5.04 mt/ha). The enhanced yields were attributed to more effective tillers per m² (260.17 tillers/m² and 253.17 tillers/m², respectively), longer panicle length (28.12 cm and 25.99 cm), more filled grains per panicle (210.03 and 215.73), and lower sterility percentage (24.93% and 26.95%). Despite comparable yields, nano urea application proved to be more cost-effective [97,926.10 Nepalese Rupees (NRs)] with a higher benefit-cost ratio (1.78) and greater ease of application for farmers compared to the LCC. Varietal responses varied, with Hardinath-1 exhibiting the highest yield with LCC-based nitrogen application (5.37 mt/ha), and Chaite-5 demonstrating the highest yield (4.778 mt/ha); with nano urea-based nitrogen application (5.31 mt/ha). Chaite-5 displayed a greater effective number of tillers per m² (241.42 tillers/m²) and filled grains per panicle (224.56). Consequently, it is suggested that nano urea-based nitrogen application, particularly in conjunction with a variety of Chaite-5, holds the potential for improved productivity.

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Introduction

Rice (*Oryza sativa* L.) is a fundamental staple diet for over 60% of the global population and is cultivated across numerous nations, with the Asia-Pacific region contributing to more than 90% of the world's rice production (Nasiruddin & Roy, 2012; Papademetriou et al., 2000). In Nepal, where it covers a substantial portion of agricultural land, rice production stands at 14.73,474 ha, contributing 11.30% to the Agricultural Gross Domestic Product (AGDP) (MoALD, 2022). Nepal holds the 17th position globally in rice production and ranks 64th in rice productivity. Over the years, rice production has seen an increase, rising from 5.047 million tons in 2013/14 to 5.62 million tons in 2020/21, accompanied by a rise in productivity from 3.39 tons/ha to 3.82 mt/ha during the same period. Despite these improvements, the production area has witnessed a decline from 1,486,951 ha in 2013/14 to 1,473,474 ha in 2020/21 (MoALD, 2022). Factors such as the introduction of high-yielding varieties and technological advancements have contributed to this growth (Dawadi et al., 2023; Ghimire & Rauniyar, 2023); however, Nepal's rice production remains comparatively low when benchmarked against neighboring countries and the global average. Spring rice varieties, known for their short growth cycle, resilience to pests and diseases, and potential for high yields, are especially promising. Planting spring rice during areas' abundant irrigation capacity helps suppress weed growth, and the increased sunlight during the spring contributes to higher yields (Subedi et al., 2018). Particularly, in regions like Nawalparasi West, Nepal, where rice cultivation thrives with suitable agro-climatic conditions and fertile soil, exploring novel approaches for optimizing nitrogen management becomes imperative.

Rice requires several essential nutrients, including nitrogen, phosphorus, potassium, calcium, magnesium, silicon, boron, iron, manganese, and zinc, for proper growth and development. Nitrogen is the most limiting nutrient, followed by phosphorus and potassium, among these elements. Efficient nitrogen fertilization plays a pivotal role in enhancing rice yields and ensuring optimal crop growth (Yadvinder-Singh et al., 2007). Nitrogen is a crucial factor in crop yield, contributing to around 20% of the total yield, highlighting the importance of proper nitrogen management in rice cultivation. Providing the necessary nutrients in appropriate amounts using the correct application methods is crucial for ensuring the productivity and sustainability of the rice cropping system (Fageria et al., 2003). However, improper fertilizer application can lead to increased costs, reduced returns, and environmental pollution (Timilsina et al., 2018), and nitrogen input levels have a significant impact on yield, biomass, harvest index, and nitrogen use efficiency (Pan et al., 2017). However, the traditional application of nitrogen fertilizer has reached a plateau up to the present. Coming from 1985 to 2010 cereal yield increased by 65% while consumption of fertilizer by 512% (Chen et al., 2011). This scenario indicates a low level of efficiency in the use of fertilizers in the crop system. The loss of nitrogen through various means, such as de-nitrification, leaching, and emissions, demands the adoption of advanced tools and practices for precise nitrogen application (Gautam et al., 2024). Loss of nitrogen can occur in various forms, such as NH_4, NO_3, or NO_2 (Ghimire, Dhami, et al., 2023; Ghimire, Poudel Chhetri, et al., 2023). Under submerged conditions, leaching losses can be as high as 80-84% (Sahu & Samant, 2006). Additionally, with increased nitrogen application rates, the volatilization of ammonia also increases in rice fields (Lin et al., 2007). Various decision support tools have been introduced to enhance nitrogen use efficiency in rice and facilitate real-time N management. These tools include the Green Seeker optical sensor, Soil Plant Analysis Development (SPAD), LCC, urea briquette, Urea Super Granules (USG), and split application (Lee, 2021). In this context, the LCC emerges as an accessible and cost-effective tool for real-time nitrogen management, particularly in South Asia (Singh et al., 2016). The introduction of tools such as the LCC provides farmers with a cost-effective means to monitor the nitrogen status of rice plants, ensuring optimal fertilization practices and, consequently, higher yields (Sathiya & Ramesh, 2009). It is an inexpensive and straightforward tool used to monitor the greenness of rice leaves, which is an indicator of the plant's nitrogen status. The LCC offers a practical tool for implementing the Site-Specific Nutrient Management (SSNM) concept, allowing farmers to make informed decisions regarding top-dressing nitrogen application in rice crops. Its applicability extends to wheat and maize, providing a visual means for farmers to assess the nitrogen requirements of their crops. Implementation of LCC resulted in a significant increase in average grain yield (0.1 to 0.7 ton/ha) across various villages and seasons (Alam et al., 2005), with notable improvements in net returns (19-31%) in rice-wheat cropping systems compared to fixed-time nitrogen application (Shukla et al., 2004). Furthermore, utilizing LCC led to a reduction in nitrogen application (20.0-42.5 kg N/ha) compared to the highest level of fixed timing nitrogen application (Maiti et al., 2004), contributing to a decrease in nitrous oxide emission by 16% and methane by 11% (Bhatia et al., 2012). Farmers can effectively employ LCC as a qualitative tool to evaluate the foliar nitrogen status of crops, guiding the application of topdressing nitrogen fertilizer as needed (Balasubramanian et al., 1998). The emergence of nanotechnology offers a sustainable solution to challenges faced by modern intensive agriculture. Nanofertilizers, falling within the 1-100 nm size range, present the potential to meet plants' nutritional needs, promoting sustainable crops (Hasanuzzaman et al., 2020). Moreover, the introduction of nanotechnology, specifically nano urea, offers a promising avenue for sustainable agriculture by addressing challenges associated with conventional fertilizers (Kim et al., 2018; Sabir et al., 2014). Given the potential benefits of nano urea, evaluating its effectiveness alongside conventional practices becomes imperative for advancing agricultural sustainability.

Nawalparasi West, situated in the Lumbini Province of Nepal, has emerged as a significant hub for rice production, leveraging its favorable agro-climatic conditions. However, the existing gap in nitrogen use efficiency and the persistent issue of suboptimal fertilization practices warrant an in-depth investigation. While rice remains a staple for more than half of the global population, the national production in Nepal struggles to meet domestic demands, particularly due to inefficiencies in nitrogen use, notably in rain-fed conditions (Baral et al., 2020). The prevalent lack of site-specific nutrient management and arbitrary fertilizer application practices contribute to a substantial yield gap in the country (Shukla et al., 2004). Unchecked fertilizer application not only results in lower crop production but also imposes environmental burdens and exacerbates the high yield gap. Compounding the issue is the reported unavailability and high cost of urea fertilizer in Nepal, further hindering effective nitrogen management. These challenges necessitate a comprehensive study to evaluate and optimize nitrogen application methods, focusing on their effectiveness, economic viability, and environmental impact. The necessity for improved nitrogen management practices, coupled with the exploration of high-yielding spring rice varieties, underscores the significance of this study in Nawalparasi West, Nepal.

The study hypothesized that employing different nitrogen management practices will significantly impact the growth and yield of spring rice varieties in Nawalparasi West, Nepal. The objective of the study was to assess the effectiveness of various nitrogen application methods on the growth and yield of spring rice and evaluate the performance of Chaite-5 and Hardinath-1 rice varieties. The research findings hold practical significance for rice cultivation in the region by informing farmers about effective nitrogen management methods to enhance crop yield and resource utilization. Furthermore, the study explored the viability of innovative approaches like nano urea, offering a potentially sustainable solution to challenges in conventional urea application, thereby providing valuable guidance for farmers, policymakers, and agricultural practitioners in Nepal to improve nitrogen management practices for sustainable and efficient rice production.

Materials and methods

Experimental Site

The experimental site for the study was the Semari farmer's plot in Pratappur, Nawalparasi West, situated at coordinates 27.53° N 83.70° E, within the Lumbini province in the Terai region of Nepal. Conducted during the spring season of 2023, the site experiences a tropical monsoon climate characterized by four distinct seasons: *i)* summer, *ii)* monsoon, *iii)* autumn, and *iv)* winter. The climate provides favorable conditions for spring rice cultivation, with the crop season typically spanning from March to May, as indicated by NASA (2023).

Physiochemical Characteristics of Soil

The physiochemical characteristics of the soil were assessed by randomly collecting samples from the plot in a 'Z' pattern, utilizing a shovel for soil excavation. Subsequently, the sub-samples were amalgamated, air-dried under shade, and ground, and then submitted to the soil testing laboratory in Bhumai, Nawalparasi West. The analysis revealed a loam soil texture for the research plot, providing the following observations (Table 1).

Experimental Materials and Experimental Details

The experimental materials for the study included Chaite-5 and Hardinath-1 rice varieties which were acquired from Buddha Seeds Company, Nawalparasi West. Additionally, nitrogen management involved LCC with 25% basal urea application, along with foliar spray of nano urea at the maximum tillering stage and before panicle initiation, serving as key components for nitrogen management in spring rice. The experimental design employed a two-factorial Randomized Complete Block Design (RCBD) with nitrogen doses (4 levels) and varieties (2 types). The study comprised 8 treatments with 3 replications (Table 2). Each plot measured 3 × 2 m², resulting in a plot size of 6 m². There was a 1 m spacing between replications and treatments. The total experimental plot size covered an area of 325 m², arranged in a 25 × 13 m layout.

General cultivation practices

Nursery management, main field preparation, and fertilizer application

The nursery management involved periodic irrigation and thorough inspection for pest and disease symptoms in Chaite-5 and Hardinath-1 spring rice varieties. The main field, previously plowed, underwent additional plowing with standing water using a rotavator to create a puddled field for rice transplantation. Nitrogen (N), Phosphorus (P), and Potassium (K) were sourced from urea/nano urea, Single Super Phosphate (SSP), and Muriate of Potash (MoP), respectively. For the experimental plots, P and K were applied as a full basal dose through SSP (150 g per plot) and MoP (39.9 g per plot) with the recommended dose 60:40 PK kg/ha (AITC, 2023). A suggested approach is to apply the fertilizer in three stages: as a basal dose, a top dressing at the maximum tillering stage, and a second top dressing at the tillering stage. Nitrogen application included five different methods. Control treatment (0 kg N/ha), where no nitrogen was applied. Nutrient dose of 120 kg N/ha: 25% N as basal dose (urea: 39.13 g per plot), 50% N in tillering stage (urea: 78.26 g per plot), and 25% during panicle initiation stage (urea: 39.13 g per plot). PAU-LCC-based nitrogen management: Initially, 25% N was applied as basal dose (urea: 39.13 g per plot), and from 14 Days After Transplanting (DAT) onwards, need-based nitrogen was applied at 30 kg/ha intervals if the plant leaves' shade fell below the critical shade of LCC. Nano urea foliar spray: Initially, 25% N was applied as a basal dose (urea: 39.13 g per plot), followed by two foliar sprays of Indian Farmers Fertilizer Cooperative Limited (IFFCO) nano urea at maximum tillering and panicle initiation stages, with a concentration of 4 ml/1 liter of water.

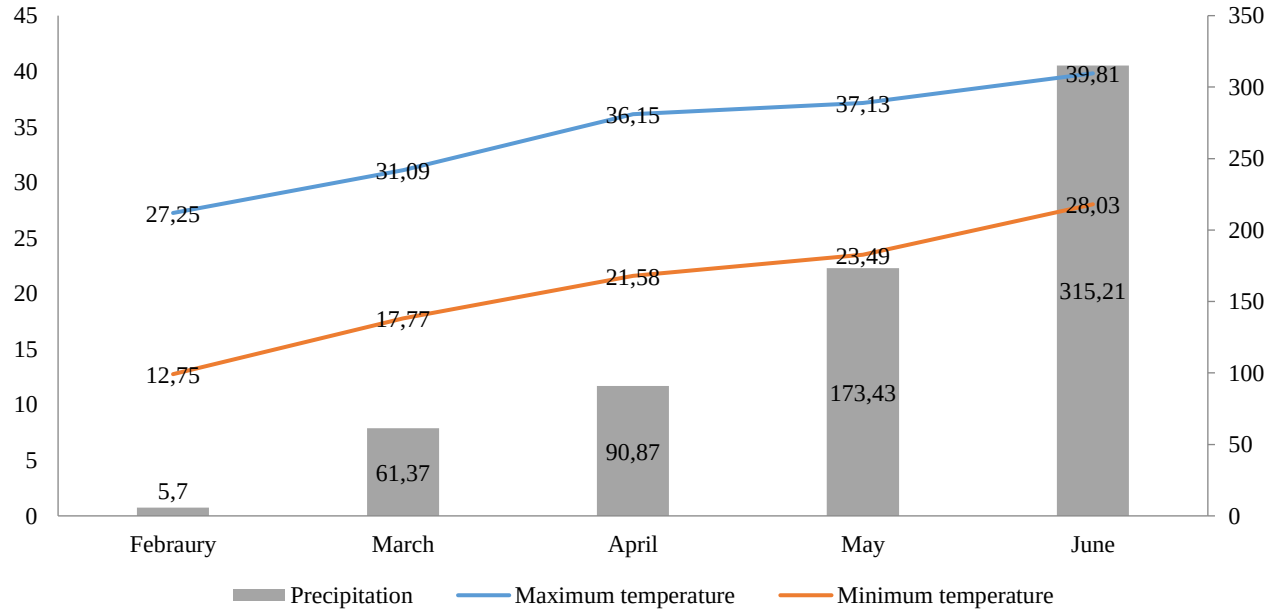

Figure 1. Maximum temperature, minimum temperature, and precipitation of Nawalparasi West from February 2023 to June 2023.

Table 1. Physiochemical properties of soil at the research site, Nawalparasi West.

S.N	Soil test	Test methods	Value
1	pH	Probe method	7.1
2	Soil organic matter (%)	Walkey and black method (Houba et al., 1989)	2.57
3	Nitrogen (%)	Kjeldahl method (Bremner & Hauck, 2015)	0.103
4	Phosphorus	Modified Olsen's method (Watanabe & Olsen, 1965)	36.23
5	Potassium	Ammonium acetate method (Pratt, 2016)	349.44
6	Soil texture	The hydrometer method (Mozaffari et al., 2024)	Loam

Table 2. Different treatment factors were used in the experiment.

Factor A: Nitrogen application methods		
1.	Control	N_1
2.	Split urea application based on RDF (120 kg/ha)	N_2
3.	Leaf color chart (PAU-LCC) based urea application with 25% basal urea application (30 kg/ha)	N_3
4.	25% RDF-based urea application (30 kg/ha) and then 2 foliar sprays of IFFCO nano urea (4 ml/l)	N_4
Factor B: Varieties		
1.	Chaite-5	V_1
2.	Hardinath-1	V_2

Transplantation of seedlings

The transplantation phase involved manual transplantation with a spacing of 20 cm x 20 cm and three seedlings per hill. Seedlings aged 30-35 days were utilized for this process. Before uprooting, the seedlings in the nursery underwent irrigation. The actual transplantation occurred promptly, within 24 hours of uprooting the seedlings, ensuring their swift and efficient relocation to the main field.

Irrigation, weed management, and plant protection measures

Rice, being a water-intensive crop, demands a substantial water supply, particularly during critical growth stages such as tillering, panicle initiation, and grain filling. To ensure an adequate water supply, tube well irrigation was diligently maintained. Weed management was addressed through hand weeding, with the initial weeding conducted at 30 DAT and the subsequent one at 45 DAT. In terms of pest and disease control, a proactive approach was adopted. Prophylactic and curative pesticide sprays were applied at appropriate doses, synchronized with the appearance of signs of diseases and pests. This strategy aimed to preemptively manage and address potential threats to the rice crop, promoting a healthy and productive cultivation environment.

Harvesting and threshing

The harvesting process involved manual cutting using traditional sickles, with special attention given to a central 3 m square area marked and harvested separately within each plot. Following harvest, the rice heads were cut, sun-dried, and manually threshed. The grains underwent a cleaning process through winnowing, and their weight was meticulously measured using an electric balance. This traditional yet meticulous approach to harvesting and threshing aimed to ensure the accurate assessment of grain yield in each experimental plot.

Data Collection

Plant height, number of tiller per square meter, and number of effective tillers per square meter

Phenological recording involved the measurement of plant height at 15-day intervals from 30 DAT for five randomly selected and tagged plants. Additionally, the quantification of tillers per square meter commenced at 30 DAT, with measurements taken at 15-day intervals throughout the crop cycle (Table 3). Effective tillers, characterized by the presence of grains, were meticulously recorded, and the count per square meter was calculated for each plot just before the crop's harvest. This systematic approach provided comprehensive data on plant height, tiller density, and effective tiller production, contributing to a detailed understanding of the experimental outcomes.

Flag leaf length and panicle length (cm)

The flag leaf length, representing the first leaf below the inflorescence in gramineous plants, was meticulously measured, specifically focusing on the first leaf beneath the panicle. Simultaneously, panicle length was assessed by randomly selecting 20 panicles from each hill, and their respective measurements were recorded as the values for panicle length. These detailed measurements provided crucial insights into the development and characteristics of the experimental rice varieties, contributing to a comprehensive evaluation of the study's outcomes.

Number of filled grains per panicle, thousand-grain weight, sterility percentage

The average number of grains was derived from 20 carefully selected samples in each plot within the experiment for the determination of the final data. These samples, representing various treatments, underwent meticulous weighing on a precision weighing machine to determine the weight of a thousand grains. Each selected panicle was scrutinized, and the number of unfilled grains per panicle was recorded to assess sterility percentage. The sterility percentage was then calculated using Equation 1. This method provided a quantitative measure of sterility, offering valuable insights into the reproductive success and overall grain quality in different treatment conditions.

$$\text{Sterility } \% = \frac{\text{Number of unfilled grains}}{\text{Total number of filled grains}} \times 100 \quad (1)$$

(Puteh et al., 2014)

Grain yield, straw yield, and harvest index

The determination of grain yield, straw yield, and harvest index involved selecting a central 1 m² plots for harvesting the crops. The harvested crops underwent sun drying, threshing, and cleaning before recording their weights. Grain moisture levels were measured using a moisture meter, and a straw sample was set aside for sun drying. The grain and straw yields obtained were then used to calculate the yields for the entire hectare area.

For grain yield, an adjustment was made to account for a moisture level of 14% using the formula proposed by Shahidullah et al. (2009), as stated in Equation 2.

$$GY = \frac{(100-MC)*\text{plot yield (kg)}\times 1000(\text{ha})}{(100-14)\times A\times 1000\ (\text{mt})} \quad (2)$$

GY: Grain yield (mt/ha) at 14% moisture

Where, MC = Moisture content of grain (%) just before weighing the bulk, and A = Net plot area (m^2).

The straw yield of the selected sample was measured in tons/ha, and the harvest index (HI) was calculated using Equation 3.

$$HI\ (\%) = \left[\frac{\text{Grain yield}}{\text{Grain yield+Straw yield}}\right] \times 100 \quad (3)$$

(Bhatt & Ghimire, 2024)

Economic analysis

The total cost of cultivation, total return, net return, and benefit-cost ratio (BCR) were calculated. The economic analysis of the experimental cultivation involved a comprehensive assessment of various financial aspects. The total cost of cultivation comprises all incurred expenses, encompassing inputs such as seeds, fertilizers, pesticides, labor, machinery usage, and irrigation. On the other hand, the total return signifies the overall revenue generated through the sale of the harvested crops, determined by multiplying the yield with the market prices. Net return, a critical metric, represents the actual profit derived from deducting the total cost of cultivation from the total return, offering insights into the financial outcome of the agricultural endeavor. Additionally, the benefit-cost (BC) ratio serves as a key indicator, quantifying the relationship between benefits and costs. A BCR exceeding 1 indicates a profitable venture, while a ratio below 1 suggests a potential financial loss. These economic analyses contribute valuable information for farmers, researchers, and policymakers to make informed decisions regarding the economic viability and sustainability of the implemented agricultural practices.

Statistical Analysis

The recorded data subjected statistical analysis, including analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) for mean separations, utilizing R-Studio 4.3.1 software. Microsoft Word 2010 was employed for word processing, and MS Excel was used for creating tables, graphs, and basic statistical analysis. ANOVA was utilized to assess differences between the two factors, and LSD values were calculated at a 5% level of significance using R-studio.

Results and Discussion

Growth Parameters

Plant height

The examination of plant height, a pivotal indicator of crop development, unfolded notable variations influenced by both nitrogen application methods and rice varieties. The numerical data presented in Table 4 provides insights into the growth dynamics at various stages. At 30 DAT, the plant height in the control plot was 48.63 cm, significantly lower than treatments involving nitrogen application. No significant difference was observed between LCC (56.58 cm), nano urea (55.80 cm), and RDF (54.41 cm) at this early stage.

Table 3. Details of various cultural practices in an experimental plot of rice.

S.N	Cultural operations	Date
1.	Seed sowing	February 11, 2023
2.	Collection of soil samples and	February 27, 2023
3.	Main field preparation	March 16, 2023
4.	Transplanting	March 17, 2023
5.	Application of basal dose of fertilizer	March 17, 2023
6.	Top dressing	
6.1	1st top dressing	April 12, 2023
6.2	2nd top dressing	May 5, 2023
7	LCC reading	
7.1	1st LCC reading	April 1, 2023
7.2	2nd LCC reading	April 11, 2023
7.3	3rd LCC reading	April 21, 2023
7.4	4th LCC reading	May 1, 2023
7.5	5th LCC reading	May 11, 2023
8	Weeding	
8.1	1st weeding	April 18, 2023
8.2	2nd weeding	May 03, 2023
9	Harvesting	June 20, 2023 (Chaite-5) June 23, 2023 (Hardinath-1)
10	Threshing	June 21, 2023 (Chaite-5) June 24, 2023 (Hardinath-1)

Table 4. Plant height as influenced by nitrogen application methods and spring rice varieties.

Treatments	Plant height (cm)				
	30 DAT	45 DAT	60 DAT	75 DAT	90 DAT
Nitrogen application methods					
Control (0 kg N/ha)	48.63^{b}	63.67^{c}	78.53^{c}	80.67^{c}	87.33^{c}
RDF (120 kg N/ha)	54.41^{a}	68.50^{b}	83.33^{bc}	88.00^{b}	95.67^{b}
30 kg N/ha + LCC	56.58^{a}	71.67^{ab}	87.83^{ab}	96.50^{a}	102.00^{ab}
30 kg N/ha + Nano urea	55.80^{a}	73.67^{a}	91.50^{a}	95.33^{a}	103.10^{a}
SEm (±)	1.78	1.14	1.76	1.87	2.25
$LSD_{0.05}$	5.39	3.46	5.35	5.67	6.83
F-test	*	***	***	***	***
Varieties					
Chaite-5	53.89^{a}	69.19^{a}	83.10^{b}	87.67^{b}	94.25^{b}
Hardinath-1	53.82^{a}	69.58^{a}	87.50^{a}	92.59^{a}	99.80^{a}
SEm (±)	1.26	0.81	1.25	1.32	1.59
$LSD_{0.05}$	3.81	2.49	3.79	4.01	4.83
CV (%)	8.08	4.03	5.07	5.08	5.69
F-test	NS	NS	*	*	*
Grand mean	53.86	69.35	85.30	93.99	97.02

Treatment means separated by DMRT and columns represented with the same letter(s) are non-significant at 5% level of significance, DAT = Days after transplanting, NS = non-significant, LSD = Least Significance Difference, SEm = Standard Error of mean, CV = Coefficient of Variance, RDF = Recommended Dose of Fertilizer, ***= significant at 0.001 level of significance, *= significant at 0.05 level of significance.

From 45 DAT onwards, the superiority of nano urea became evident, consistently exhibiting the highest plant height: 73.67 cm at 45 DAT, 91.50 cm at 60 DAT, 95.33 cm at 75 DAT, and 103.10 cm at 90 DAT. LCC showed comparable growth, while RDF followed, and the control consistently displayed the lowest plant height. At 30 and 45 DAT, Chaite-5 and Hardinath-1 showed similar plant heights. However, from 60 DAT onward, Hardinath-1 exhibited significantly greater plant height: 87.50 cm at 60 DAT, 92.59 cm at 75 DAT, and 99.80 cm at 90 DAT, compared to Chaite-5.

The early-stage uniformity in plant height across nitrogen application methods suggests that initial growth responses were comparable. Nano urea emerges as a promising option for farmers, demonstrating consistent growth benefits throughout the crop cycle. The observed increase in plant height in the nano urea and LCC treatments can be elucidated by the distinctive mechanisms associated with these nitrogen management approaches. Nano urea, characterized by the encapsulation of urea in nanocarriers, facilitates efficient nutrient release, ensuring a sustained and controlled nitrogen supply to the plants (Iqbal et al., 2019). This leads to continuous and robust vegetative growth, contributing to elevated plant height. Additionally, nano urea's targeted nutrient delivery system enhances nutrient uptake efficiency, further promoting optimal growth conditions (Midde et al., 2022). Reduced nitrogen loss from the soil due to minimized leaching and volatilization in nano urea formulations also contributes to increased plant height (Dimkpa et al., 2020). Similarly, the leaf color chart enables precision in nitrogen application by visually indicating the crop's nitrogen status. The enhanced plant height associated with LCC aligns with its positive impact on meristematic development, cell division, and cell elongation, as reported in previous studies (Bhavana et al., 2020). This allows farmers to adjust nitrogen application timely and accurately, ensuring that the plants receive an optimal amount of nitrogen for healthy and vigorous growth, including the observed higher plant height. The combination of these factors highlights the effectiveness of nano urea and LCC in promoting enhanced plant height through improved nutrient management strategies. Varietal differences became more pronounced in later stages, with Hardinath-1 showcasing taller plants, emphasizing the impact of specific rice varieties on growth dynamics.

Number of tillers per square meter

The number of tillers per square meter in spring rice crops, recorded from 30 to 90 DAT at 15-day intervals, exhibited significant variations based on nitrogen management methods and rice varieties. The data revealed a continuous increase in tiller numbers up to 60 DAT, followed by a decline thereafter. At 30 DAT, no significant difference was observed among nitrogen management methods (Nano urea, LCC, and RDF), but by 45 DAT, LCC exhibited the highest number of tillers per square meter (300.50), statistically comparable to nano urea (293.67) and RDF (291.50). Nano urea consistently demonstrated the maximum number of tillers per square meter at 60 (509.13), 75 (379.50), and 90 DAT (287.00), surpassing other treatments (Table 5). The control group exhibited the lowest tiller count at all observed time points (212.83 to 217.17). This outcome suggests that both nano urea and LCC are effective in enhancing the number of tillers, aligning with findings from Adhikari et al. (2022). Furthermore, the influence of rice varieties on tiller numbers was evident, with Hardinath-1 initially exhibiting higher tiller counts at 30 DAT (281.42). However, from 45 DAT onward, Chaite-5 consistently displayed significantly higher tiller numbers (374.41 to 268.33) compared to Hardinath-1. This observation aligns with results reported by Shrestha et al. (2022), indicating a varietal difference in tillering patterns. Overall, the enhanced tiller numbers associated with nano urea and LCC can be attributed to their precise and effective nitrogen management, promoting optimal vegetative growth (Midde et al., 2022). The results underscore the significance of these nitrogen management strategies in influencing tillering dynamics in spring rice cultivation.

The higher tillering observed in the LCC and nano urea foliar application treatments can be attributed to the efficient and site-specific nitrogen management strategies employed by these methods. LCC is a visual tool that enables farmers to assess the nitrogen status of crops and make informed decisions about nitrogen application (Ali et al., 2017). It allows for real-time adjustments based on the visual indicators of nitrogen deficiency or sufficiency in plant leaves. In the case of nano urea foliar application, the use of nanotechnology in delivering nitrogen to plants offers several advantages. Nano urea provides a more controlled and targeted release of nitrogen, ensuring that the nutrient is efficiently taken up by the plants when needed (Vejan et al., 2021). This precision in nutrient delivery can lead to optimal conditions for tillering, promoting robust vegetative growth. Both LCC and nano urea foliar application methods contribute to a more tailored and responsive approach to nitrogen management, which likely results in an environment conducive to higher tillering. These approaches align with the concept of Site-Specific Nutrient Management (SSNM), where nutrient application is customized based on the specific needs of the crop at different growth stages. The ability to fine-tune nitrogen application in response to the crop's requirements may lead to the observed higher tillering in the LCC and nano urea treatments compared to conventional methods.

Yield Attributing Parameters

The study delved into various yield attributes influenced by different nitrogen application methods and rice varieties, shedding light on their intricate impact on rice growth and productivity.

Table 5. Number of tillers/m^2 as influenced by nitrogen application methods and spring rice varieties.

Treatments	Number of tillers m^{-2}				
	30 DAT	45 DAT	60 DAT	75 DAT	90 DAT
Nitrogen application methods					
Control (0 kg N/ha)	212.83^{b}	328.50^{c}	423.17^{c}	317.83^{c}	217.17^{c}
RDF (120 kg N/ha)	291.50^{a}	365.00^{b}	487.50^{b}	355.00^{b}	258.83^{b}
30 kg N/ha + LCC	300.50^{a}	388.33^{a}	506.17^{a}	377.00^{a}	273.50^{ab}
30 kg N/ha + Nano urea	293.67^{a}	377.67^{ab}	509.13^{a}	379.50^{a}	287.00^{a}
SEm (±)	4.86	7.051	6.11	6.61	8.60
$LSD_{0.05}$	14.76	21.39	18.53	20.06	26.10
F-test	***	***	***	***	***
Varieties					
Chaite-5	267.83^{b}	374.41^{a}	488.58^{a}	366.25^{a}	268.33^{a}
Hardinath-1	281.42^{a}	355.33^{b}	474.50^{b}	348.42^{b}	249.42^{b}
SEm (±)	3.44	4.98	4.32	4.67	6.08
$LSD_{0.05}$	10.43	15.12	13.10	14.19	18.46
CV (%)	4.34	4.73	3.11	4.53	8.14
F-test	*	*	*	*	*
Grand mean	274.63	364.87	481.54	357.33	258.87

Treatment means separated by DMRT and columns represented with the same letter(s) are non-significant at a 5% level of significance, DAT = Days after transplanting, LSD = Least Significance Difference, SEm = Standard Error of mean, CV = Coefficient of Variance, RDF = Recommended Dose of Fertilizer, ***= significant at 0.001 level of significance, *= significant at 0.05 level of significance.

Table 6. Yield attributing characters as influenced by nitrogen application methods and spring rice varieties.

Treatments	Yield attributes					
	ET/m^2	Flag leaf length (cm)	Panicle length (cm)	FGPP	TGW (g)	Sterility (%)
Nitrogen application methods						
Control (0 kg N/ha)	191.33^{c}	25.33^{c}	21.75^{c}	159.77^{c}	23.17^{a}	30.22^{a}
RDF (120 kg N/ha)	223.00^{b}	28.00^{bc}	22.82^{c}	189.93^{b}	24.10^{a}	23.45^{b}
30 kg N/ha + LCC	260.17^{a}	30.90^{ab}	28.12^{a}	210.03^{a}	24.27^{a}	24.93^{b}
30 kg N/ha + Nano urea	253.17^{a}	33.67^{a}	25.99^{b}	215.73^{a}	24.12^{a}	26.95^{ab}
SEm (±)	9.33	1.12	0.54	4.15	0.79	1.40
$LSD_{0.05}$	28.31	3.41	1.64	12.58	2.38	4.27
F-test	***	***	***	***	NS	*
Varieties						
Chaite-5	241.42^{a}	28.87^{b}	21.58^{b}	224.56^{a}	20.14^{b}	28.37^{a}
Hardinath-1	222.42^{a}	30.87^{a}	27.76^{a}	163.17^{b}	27.68^{a}	24.39^{b}
SEm (±)	6.60	0.80	0.38	2.93	0.55	0.99
$LSD_{0.05}$	20.01	2.41	1.16	8.89	1.68	3.02
CV (%)	9.86	9.34	5.37	5.24	8.04	13.08
F-test	NS	*	***	***	***	*
Grand mean	231.92	29.47	24.67	193.87	23.91	26.39

Treatment means separated by DMRT and columns represented with the same letter(s) are non-significant at a 5% level of significance, NS = non-significant, LSD = Least Significance Difference, SEm = Standard Error of mean, CV = Coefficient of Variance, RDF = Recommended Dose of Fertilizer, *** = significant at 0.001 level of significance, * = significant at 0.05 level of significance.

Table 7. Interaction effect of nitrogen application methods and rice varieties on panicle length.

Treatment combinations	Panicle length (cm)	
	Varieties	
Nitrogen application methods	Chaite-5	Hardinath-1
Control (0 kg N/ha)	18.50[d]	24.99[c]
RDF (120 kg N/ha)	20.43[d]	25.21[c]
30 kg N/ha + LCC	23.24[c]	33.00[a]
30 kg N/ha + Nano urea	24.15[c]	27.84[b]
SEm (±)	0.77	
$LSD_{0.05}$	2.32	
CV (%)	5.64	
F-test	**	
Grand Mean	24.67	

Treatment means separated by DMRT and columns represented with the same letter(s) are non-significant at 5% level of significance, LSD = Least Significance Difference, SEm = standard Error of mean, CV = Coefficient of Variance, ** = significant difference at 0.01 level of significance, RDF = Recommended Dose of Fertilizer.

In terms of effective tillers per square meter (ET/m^2), the treatment with 30 kg N/ha + LCC emerged as the most effective, showcasing the importance of innovative nitrogen management methods in promoting tiller development. Conversely, the control treatment exhibited the lowest ET/m2, emphasizing the necessity of proper nitrogen utilization for optimal tillering (Table 6). The results of a higher number of effective tillers per m^2 in LCC-based nitrogen management were in harmony with Adhikari et al. (2022). Moving to flag leaf length, the treatment with 30 kg N/ha + Nano urea stood out with the longest flag leaf (33.67 cm), indicating the positive influence of nano urea on leaf development. In contrast, the control treatment showed the shortest flag leaf (25.33 cm), emphasizing the pivotal role of nitrogen in supporting healthy plant growth (Table 6).

When considering panicle length, 30 kg N/ha + LCC exhibited the longest panicle (28.12 cm), highlighting the positive impact of LCC-based nitrogen management on panicle elongation (Table 6). Both 30 kg N/ha + Nano urea and RDF treatments also demonstrated competitive panicle lengths, underscoring the effectiveness of these nitrogen sources. The interaction between nitrogen application methods and rice varieties significantly influenced panicle length, revealing distinct patterns in panicle development across different treatment combinations (Table 7). The treatment combination of 30 kg N/ha + LCC and Hardinath-1 demonstrated the longest panicle length at 33.00 cm, indicating a synergistic effect between LCC-based nitrogen management and the specific rice variety. Conversely, the other treatment combinations, including Control (0 kg N/ha), RDF (120 kg N/ha), and 30 kg N/ha + Nano urea, produced significantly shorter panicles. This result underscores the importance of considering both nitrogen management practices and rice varieties for optimizing panicle development. The observed longer panicle length in the treatment combining LCC and Hardinath-1 suggests potential compatibility or positive interaction between the unique characteristics of Hardinath-1 and the benefits derived from LCC-based nitrogen application. The contribution and variation in crops are primarily led by their genotypes. Genotypes represent the genetic makeup of a crop, and the variation in traits such as growth and yield arises from different combinations of these genotypic factors (Ghimire, Neupane, et al., 2023). The diverse genotypic combinations contribute to the overall variability observed in crop characteristics, influencing their performance and adaptability to various environmental conditions.

In terms of filled grains per panicle (FGPP) and thousand-grain weight (TGW), 30 kg N/ha + Nano urea (215.73 FGPP and 24.12 g, respectively) consistently outperformed other treatments, indicating enhanced grain development and maturation. This observed enhancement in grain filling can be attributed to the unique properties of nano urea. The nano-sized particles facilitated a more efficient translocation of starch from the actively photosynthesizing areas of the leaves and straw toward the developing grains (Basavegowda & Baek, 2021). This increased translocation efficiency is associated with the smaller particle size of nano urea, allowing for better mobility within the plant's vascular system. Furthermore, the application of nano urea contributed to an elevated and sustained nitrogen supply during critical growth stages (Upadhyay et al., 2023). This sustained nitrogen availability facilitated an extended period of enhanced photosynthetic activity. According to findings by Sahu et al. (2022), nano urea has been reported to provide a continuous and controlled release of nitrogen, ensuring a steady nutrient supply to the plants. This prolonged availability of nitrogen played a crucial role in optimizing the interception of sunlight for photosynthesis, thereby promoting greater overall photosynthetic efficiency. In essence, the combined effects of improved starch translocation and sustained nitrogen availability induced by nano urea application synergistically contributed to the observed increase in the number of filled grains per panicle. This underscores the potential of nano urea not only in inefficient nutrient delivery but also in positively influencing the physiological processes crucial for achieving optimal crop yield.

The lower sterility percentage in treatments with 30 kg N/ha + LCC (24.93%) and 30 kg N/ha + Nano urea (26.95%) further attested to the positive impact of these innovative nitrogen management methods on grain quality. Moreover, varietal differences were evident, with Chaite-5 consistently outperforming Hardinath-1 in most yield attributes, emphasizing the importance of selecting suitable rice varieties for optimal yields. The distinctive trends observed in various agronomic parameters, including effective tillers per square meter, flag leaf length, panicle length, FGPP, TGW, and sterility, can be ascribed to the

nuanced and specific mechanisms associated with each nitrogen management method and nano urea. Beginning with the effective tillers per square meter, the LCC-based nitrogen management likely played a pivotal role in optimizing nutrient availability during critical growth stages. By offering real-time assessments and facilitating precise nitrogen applications, LCC ensured an optimal balance for tiller development, thereby resulting in a higher number of effective tillers. On the other hand, the nano-sized urea particles in nano urea might have contributed to controlled and sustained nitrogen release, fostering effective tiller development through enhanced nutrient uptake efficiency. The nano urea application might have demonstrated its efficacy by promoting efficient nutrient absorption, which translated into robust vegetative growth, including longer flag leaves (Javed et al., 2022). Simultaneously, the continuous monitoring and responsive nitrogen management offered by LCC may have maintained an optimal nutrient balance, influencing the elongation of flag leaves (Bijay-Singh & Singh, 2017). In terms of panicle length, both nitrogen management methods showed their impact. The precision in nitrogen application guided by LCC likely supported sustained panicle elongation, resulting in longer panicles. Similarly, the nano-sized urea particles in nano urea contributed to a consistent nitrogen supply during crucial growth stages, leading to increased panicle length. Moving on to the grain-related parameters, namely FGPP, TGW, and sterility percentage, both nano urea and LCC demonstrated positive effects. The controlled release of nitrogen from nano urea and the dynamic monitoring offered by LCC-optimized grain-filling processes resulted in higher FGPP and TGW. Moreover, the efficient nitrogen supply may have reduced stress conditions, contributing to lower sterility rates in both nano urea and LCC treatments.

Measurement of Yields

The grain yield, a critical parameter in determining the success of rice cultivation, exhibited significant variations under different nitrogen management methods and across rice varieties. Notably, LCC-based nitrogen management emerged as the most effective, yielding the highest grain production at 5.18 mt/ha. Nano urea application also demonstrated promising results, closely trailing behind LCC with a grain yield of 5.04 mt/ha, surpassing the conventional RDF treatment (4.45 mt/ha). This result aligns with previous findings by Krishnakumar and Haefele (2013) and Duttarganvi et al. (2014), indicating the potential of LCC in enhancing nitrogen use efficiency and consequently boosting grain yield. Varietal differences played a significant role in influencing grain yield, with Chaite-5 showcasing superior performance at 4.778 mt/ha compared to Hardinath-1 at 4.368 mt/ha (Table 8). The higher yield in Chaite-5 could be attributed to its significantly higher number of effective tillers per square meter and filled grains per panicle (Table 7). Further dissecting the interaction between nitrogen management methods and rice varieties on grain yield, LCC-based nitrogen application on Hardinath-1 rice displayed the highest yield. Intriguingly, this was statistically comparable to nano urea on Chaite-5, LCC on Chaite-5, and RDF urea on Chaite-5, suggesting nuanced varietal responses to specific nitrogen management approaches.

The interaction effect of nitrogen (N) application methods and spring rice varieties on grain yield unveils nuanced responses within specific treatment combinations. Table 9 outlines the grain yield (mt/ha) for various nitrogen application methods (Control, RDF, LCC, and Nano urea) under two rice varieties, Chaite-5 and Hardinath-1. In the control group, both varieties, Chaite-5 and Hardinath-1, exhibited relatively lower grain yields, marked as 3.997 mt/ha and 3.200 mt/ha, respectively. The RDF treatment, representing the conventional recommended dose of fertilizer, showcased an improvement in grain yield for both varieties.

Chaite-5 recorded 4.773 mt/ha, while Hardinath-1 exhibited 4.133 mt/ha. The introduction of innovative nitrogen management methods, such as Leaf Color Chart (LCC) and Nano urea, further elevated grain yields. Chaite-5, under LCC, demonstrated a notable increase to 5.020 mt/ha, while Hardinath-1 reached 5.357 mt/ha. Nano urea application resulted in even higher yields, with Chaite-5 at 5.313 mt/ha and Hardinath-1 at 4.780 mt/ha.

Table 8. Effect of different nitrogen application methods and spring rice varieties of rice on yield parameters.

Treatments	Yield parameters		
	Grain yield (mt/ha)	Straw yield (mt/ha)	Harvest index (%)
Nitrogen application methods			
Control (0 kg N/ha)	3.59^{c}	5.27^{c}	0.41^{a}
RDF (120 kg N/ha)	4.45^{b}	6.46^{b}	0.41^{a}
30 kg N/ha + LCC	5.19^{a}	7.28^{a}	0.41^{a}
30 kg N/ha + Nano urea	5.05^{a}	7.29^{a}	0.42^{a}
SEm (±)	0.14	0.12	0.01
$LSD_{0.05}$	0.418	0.37	0.25
F-test	***	***	NS
Varieties			
Chaite-5	4.778^{a}	6.961^{a}	0.408^{a}
Hardinath-1	4.368^{b}	6.194^{b}	0.411^{a}
SEm (±)	0.0975	0.0862	0.006
$LSD_{0.05}$	0.296	0.261	0.018
CV (%)	7.391	4.538	5.046
F-test	*	***	NS
Grand mean	4.572	6.578	0.409

Treatment means separated by DMRT and columns represented with the same letter(s) are non-significant at a 5% level of significance, NS = non-significant, LSD = Least Significance Difference, SEm = Standard Error of mean, CV = Coefficient of Variance, RDF = Recommended Dose of Fertilizer, *** = significant at 0.001 level of significance, * = significant at 0.05 level of significance.

Table 9. Interaction effect of N application methods and spring rice varieties on grain yield.

Treatment combinations	Grain yield (mt/ha)	
	Varieties	
Nitrogen application methods	Chaite-5	Hardinath-1
Control	3.997[c]	3.200[c]
RDF	4.773[a]	4.133[b]
LCC	5.020[a]	5.357[a]
Nano urea	5.313[a]	4.780[a]
SEm (±)	0.195	
$LSD_{0.05}$	0.592*	
CV (%)	7.390	
Grand Mean	4.572	

Treatment means separated by DMRT and columns represented with the same letter(s) are non-significant at 5% level of significance, NS= non-significant, LSD=Least Significance Difference, SEm= Standard Error of mean, CV= Coefficient of Variance, *= significant difference at 0.05 level of significance, RDF= Recommended Dose of Fertilizer.

Table 10. Cost of cultivation, total return, net return, and BCR as influenced by nitrogen application methods and spring rice varieties.

Treatments	Economic analysis			
	Total cost of production (NRs)	Total return (NRs)	Net return (NRs)	BCR
Nitrogen application methods				
Control (0 kg N/ha)	95066.10[d]	120976.00[a]	25909.87[c]	1.27[c]
RDF (120 kg N/ha)	101566.10[a]	149721.10[b]	48154.97[b]	1.47[b]
30 kg N/ha + LCC	99191.10[b]	169668.90[a]	70477.83[a]	1.71[a]
30 kg N/ha + Nano urea	97926.10[c]	174431.80[a]	76505.67[a]	1.78[a]
SEm (±)	62.50	4637.49	4615.32	0.05
$LSD_{0.05}$	189.58	14066.40	13999.13	0.14
F-test	***	***	***	***
Varieties				
Chaite-5	98499.85[a]	160563.50[a]	62063.67[a]	1.63[a]
Hardinath-1	98374.85[a]	146835.50[b]	48460.59[b]	1.49[b]
SEm (±)	44.19	3279.21	3263.52	0.03
$LSD_{0.05}$	134.05	9946.45	9898.87	0.10
CV (%)	0.15	7.39	20.46	7.35
F-test	NS	*	*	*
Grand mean	98437.35	153699.40	55262.08	1.56

Treatment means separated by DMRT and columns represented with the same letter(s) are non-significant at a 5% level of significance, ns = non-significant, LSD = Least Significance Difference, SEm = Standard Error of mean, CV = Coefficient of Variance, LCC = Leaf Color Chart, RDF = Recommended Dose of Fertilizer, *** = significant at 0.001 level of significance, * = significant at 0.05 level of significance, 1 USD = 132.37 NRs.

Moving to straw yield, it demonstrated sensitivity to both nitrogen management and rice varieties. Nano urea application resulted in the highest straw yield at 7.295 mt/ha, closely followed by LCC at 7.282 mt/ha (Table 8). These outcomes underline the importance of optimal nitrogen use in promoting vegetative growth and dry matter production. Harvest index, a critical indicator of the allocation of resources between grain and straw, did not exhibit significant differences across nitrogen management methods or rice varieties. However, the nano urea application method resulted in the highest HI (0.42). This implies that the distribution of assimilates to grain and straw components remained relatively consistent under the diverse conditions tested. The higher yield observed in nano urea and LCC treatments can be attributed to several scientific factors related to nitrogen management and physiological responses in rice plants. Both nano urea and LCC treatments involve more precise and efficient nitrogen management compared to the control and conventional RDF methods. Nano urea, being in nanoparticle form, likely provides a more controlled and sustained release of nitrogen, ensuring a consistent nutrient supply during critical growth stages. The higher yield in nano urea may be linked to improved photosynthetic efficiency (Upadhyay et al., 2023). Nano urea, as previously mentioned, likely contributes to increased interception of sunlight for photosynthesis due to sustained nitrogen availability (Astaneh et al., 2021). Similarly, LCC-guided nitrogen application ensures that the plants receive the necessary nutrients when they are most needed, optimizing the photosynthetic process and ultimately leading to higher grain yields (Yadav et al., 2017). The results indicate that nano urea foliar application treatments led to an increase in the number of filled grains per panicle (Table 7). This could be attributed to more efficient starch translocation from the leaves and straw to the developing grains. Nano urea, with its smaller particle size, may facilitate better mobility within the plant's vascular system, aiding in the translocation of starch (Seleiman et al., 2020). LCC-guided nitrogen application ensures that the plants receive sufficient nutrients, which is crucial for proper grain filling. The observed increase in yield in nano urea and LCC treatments may be indicative of optimized nitrogen use efficiency. Efficient nitrogen use is crucial for maximizing crop yield, and both nano urea and LCC are associated with improved nitrogen utilization. Nano urea's

controlled release and LCC's real-time monitoring likely contribute to a more judicious and effective use of nitrogen by the rice plants. The higher yield in nano urea and LCC treatments can be attributed to their ability to provide a more controlled and sustained nitrogen supply, enhance photosynthetic efficiency, improve starch translocation, and optimize nitrogen use efficiency.

Economic Analysis

The economic analysis of rice cultivation, as presented in Table 10, sheds light on the impact of nitrogen application methods and rice varieties on various financial parameters. Notably, treatments involving innovative nitrogen application methods, such as LCC and nano urea, showcased superior economic performance compared to conventional practices. The treatment combining 30 kg N/ha with LCC exhibited the highest total return (169,668.90 NRs; 1 USD = 132.37 NRs) and net return (70,477.80 NRs), resulting in a favorable BCR of 1.71. Similarly, the 30 kg N/ha with Nano urea treatment demonstrated significant economic gains with a BCR of 1.78. Conversely, the control group incurred lower costs but yielded a substantially reduced return and net profit. The influence of rice varieties on economic outcomes was evident, with Chaite-5 exhibiting higher returns and net profits compared to Hardinath-1.

Conclusion

The study revealed significant variations in growth parameters such as plant height and the number of tillers per m^2, as well as yield attributing characters, including effective tillers per m^2, flag leaf length, panicle length, filled grains per panicle, thousand-grain weight, and sterility percentage. These differences were observed under varying nitrogen management strategies and spring rice varieties. Notably, nano urea-based nitrogen management demonstrated superior effectiveness compared to both the recommended dose of urea application and LCC-based urea application, taking into account both yield and cost efficiency. The findings underscore the potential of nano urea as a promising alternative for improved nitrogen management practices, emphasizing the need for further research to validate and optimize its application in diverse agricultural settings. The results also revealed the commendable performance of both Hardinath-1 and Chaite-5 varieties in terms of growth and yield. Notably, Chaite-5 exhibited a higher grain yield compared to Hardinath-1. Continued investigations into nano urea's potential and its integration into agricultural systems are warranted to refine and promote its adoption as a superior nitrogen management strategy.

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Author contribution statement

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Conflict of interest

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The Impact of Food Safety Criteria on Fruit and Vegetable Exports from Türkiye to The European Union

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ABSTRACT

Food safety has become an increasingly important issue as people become more concerned about access to healthy food. Particularly in affluent societies such as the European Union, the increasing consumption of unhealthy fruits and vegetables and carcinogenic residues are constantly on the agenda. Reducing aflatoxin levels in dried foods to below health risk levels, eliminating them from food and ensuring access to healthy food are essential for food safety and human health. In this study, the impact of food safety practices in fruit and vegetable trade between the European Union and Türkiye was examined using mandarins, one of Türkiye's main fresh fruit and vegetable exports, and the European Union Rapid Alert System for Food and Feed (RASFF) notifications for food and feed for the period between 2019 and 2022. The reasons for these notifications and the requests made in this context were examined and a TOWS analysis matrix was created based on the findings obtained. In conclusion, residue and aflatoxin inspections should be included in traceability activities in Türkiye. Producers need to be informed to ensure the effectiveness of inspections. It is crucial to provide adequate support to producers to improve storage conditions for perishable and dry products and to encourage the use of the latest production techniques. It is of great importance to raise awareness of these techniques among producers. Thus, the European Union can be an alternative market to the Russian Federation, which is Türkiye's largest trading partner.

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Introduction

With the increase in the human population, which has reached eight billion people, it has become necessary to increase agricultural production in the world. In this context, there is an increase in the amount of synthetic fertilisers and pesticides used with the developing modern agriculture. This situation has become a threat to human health and food safety measures have increased rapidly in recent years, especially in developed countries. Some food safety problems have led to the emergence of important trade problems within the scope of the World Trade Organisation (WTO). In international trade, the role of food safety measures taken by countries in shaping international competition is quite high (Xiong and Summer, 2013).

Commercially produced citrus fruits in Türkiye are oranges, mandarins, lemons and grapefruit, and the species called citrus is not produced commercially due to its bitter taste and is generally used as rootstock for other citrus fruits (Güven, 2010).

The issue of food sufficiency dates back to Malthus' "An Essay on the Principle of Population of 1798" (Rosegrant and Cline, 2003). Malthus stated that the problem of food insufficiency would arise due to the geometric increase in human population and the arithmetic increase in food resources, and that the strong would continue to live as a result of natural selection (Akdoğan Gedik, 2020).

In The State of Food Insecurity in The World (FAO, 2001) report, the Food and Agriculture Organisation of the United Nations (FAO) defined food security as "the continuous physical and economic access of all people to sufficient, healthy, reliable and nutritious food in order to meet their nutritional needs and food priorities necessary for an active and healthy life at all times". This definition, which also includes food security within the concept of food security, is the definition used today (Koç and Uzmay, 2015).

Food security became an international problem in the 1940s due to the negative effects of the Second World War on agriculture and food production on a global scale. In the Universal Declaration of Human Rights, it was stated that access to food is a fundamental human right and the foundations of the "food security" approach were laid (Koç and Uzmay, 2022).

Food security is a multi-layered concept and has four basic layers. These are availability and stability, accessibility, availability, and sustainability (CFS, 2009). For food security to be fully achieved, all four dimensions must be realised at the same time (FAO, 2008). These are briefly explained (Kıymaz and Şahinöz, 2010):

- Availability of food: Sufficient quantities of food should be consistently available.
- Access to food: Sufficient economic resources must be available to access nutritious and appropriate food.
- Food utilisation: Appropriate utilisation should be based on basic knowledge of nutrition and health, as well as access to adequate water and sanitation.
- Food Sustainability: Food security should be sustained and the obstacles to it should be removed.

Food security is a concept that has gained importance in recent years. Increasing population, disruptions in food supply make the need for access to healthy food and food security practices mandatory. Due to the rapidly spreading globalisation trend in the world, competition in international markets is increasing. Countries have entered into various economic integration movements both to protect themselves from intense competition and to benefit more from the process of integration with the world. These important changes in world trade have led to changes in foreign trade policies of countries (Uyar, 2000). According to the classical economic theory, each country mutually benefits from international trade (Kara, 2005). Therefore, development is realised through trade and regional economic cooperation, which have an impact on the development of relations between countries (Terin et al., 2012). Food safety has also gained importance within the framework of the intensity of existing cooperation and the benefits it brings.

Sanitary measures include measures applied in food production, processing and distribution to reduce risks to human health. These include controls and inspections to ensure that food meets hygienic standards and can be consumed in a healthy and safe manner. SPS is regulated under the "Sanitary and Phytosanitary Measures Agreement" (SPS Agreement), an agreement established by the World Trade Organisation (WTO). This agreement regulates the application of sanitary and phytosanitary measures between WTO member countries and ensures fair and scientifically based measures in international trade.

It is envisaged that the member countries of the World Trade Organisation will regulate national measures on food safety in accordance with international standards. This approach constitutes the legal basis of good agricultural practices. As a result of increasing environmental awareness in the European Union, European Food Retailers prepared the GLOBALGAP (EUREPGAP) Protocol in 1997 (Bayraktar and Saner, 2016). The items listed here define the minimum standards accepted by leading European retailers. In 2007, the logo and name of EUREPGAP was changed and it was named GLOBALGAP (Gündüz, 2002).

In Türkiye, Good Agricultural Practices (GAP) is a system that is more known, used and preferred by both producers and consumers than GLOBALGAP (Eraktan, 2017). However, with the COVID-19 pandemic, it is predicted that the transition to internationally recognised GLOBALGAP practices will also increase in Türkiye (Akbudak and Şen, 2021). The most widely used GLOBALGAP standard is the Integrated Farm Assurance (IFA) standard, which applies to fruit and vegetables, aquaculture, floriculture, animal husbandry and more. This standard is also the basis for the GLOBALGAP Number (GGN) label, a consumer label for certified, responsible agriculture and transparency (Anonymous 1).

Initially, certification was carried out in the production of fresh vegetables and fruits and cut flowers, but today certification is carried out in three different areas and in many products (Öner Aba, 2018).

- Within the scope of plant production (fruit and vegetables, flowers and ornamental plants, field products, plant production materials, tea, etc.)
- Within the scope of animal production (cattle, calves, sheep, dairy farming, pigs, poultry, Türkiyes, etc.)
- Within the scope of aquaculture (fish species, crustaceans, molluscs, etc.)

Large retailer groups in the European Union have gathered the minimum standards required for agricultural products grown in their own countries or imported from abroad under the name GLOBALGAP in order to ensure the consumption of healthy and quality products. In addition, GLOBALGAP also encourages the implementation of the HACCP system and supports its principles (Kızılaslan and Yalçın, 2012). Food safety is an indispensable part of food quality. Therefore, every approach to ensure food safety will also affect quality. Hazard Analysis and Critical Control Points (HACCP), which is basically a food safety approach, can also be considered as a quality assurance system (Topoyan, 2003). The aim of the HACCP system is to determine the biological, chemical and physical hazard points that may cause contamination starting from raw materials and components in food production and to produce a safe food by controlling them (Boyacıoğlu, 1998).

Food safety refers to the whole set of measures taken to eliminate physical, chemical, biological and all kinds of damages that may occur in foods (Koç and Uzmay, 2015). According to the report published by FAO and World Health Organisation (WHO) in 2003, food safety refers to "all hazards that can make food chronically or acutely harmful to the health of consumers" (FAO and WHO, 2003). Although a conceptual distinction between food safety and food safety is expressed and defined in this way, the concept of food safety in the literature can be used to mean or include food safety (Topçu, 2019).

Türkiye's largest trade partner in the trade of fruit and vegetable is the Russian Federation. In 2022, Türkiye exported a total of 1.905.283.000 USD worth of products to the Russian Federation, of which 274.393.000 USD was made through mandarins. In the light of this result, Russia is the country to which Türkiye exports the most mandarins. European Union is also an important trade partner of Türkiye. Based on mandarin, when Türkiye's

mandarin exports to the European Union are analysed, it is understood that there is an export volume of approximately 37.000.000 USD. In this context, Russia can be seen as the first market for the mandarin market and the European Union can be seen as another substitute market for the Russian market.

Mandarin exports constitute a significant portion, 6.02%, of the fruit and vegetable trade from Türkiye to the European Union (EU). In light of this, it is conducted a TOWS analysis to examine the impact of EU food safety criteria, practices, and monthly RASFF notifications on this trade. The analysis focused on the RASFF notifications between 2019 and 2022, specifically those related to mandarins, and it is evaluated the mandarin trade between the European Union and Türkiye based on this information. Policies were established based on the strategies identified following the TOWS analysis, which were tabulated.

Food Safety and Mandarin Exports

Applications in Food Safety

Looking more closely at the issue of food safety, it is possible to see examples of food controls and standards for the health of consumers in the past. However, both the threats in food and the measures taken against them have increased over time. In this period of significant increases in yield and production, called the green revolution, the ecological balance began to deteriorate as a result of unconscious and excessive use of chemical fertilisers or pesticides; negative effects on the environment and human health have emerged. For this reason, the concepts of sustainable agriculture and food safety have started to be discussed with increasing consumer awareness in developed countries (Topçu, 2019).

The main purpose of the General Agreement on Tariffs and Trade (GATT), which was signed in 1947 with the participation of 23 countries, was to make progress towards the liberalisation of world trade and to discipline international trade with a different mindset. In this context, mutual and gradual reduction of tariffs and harmonisation of customs regulations, removal of non-tariff barriers or their transformation into tariffs (tariffication) were aimed (Akman and Yaman, 2008). Since 1947, it was aimed to advance this liberalisation through negotiations. As a result of the Uruguay Round Negotiations, the World Trade Organisation (World Trade Organisation) was established to replace the GATT with the Marrakesh Agreement of 1994. The European Union and many non-member countries of the European Union are also parties to this agreement.

EU, one of the important actors in the export and import of world agricultural products, has established the RASFF in order to ensure that the food products it imports are safe. Although not a member of RASFF, many countries are affected by this system due to their exports of agricultural and food products to the EU (Çebi and Olhan, 2017). Regulation No. 178/2002 of the Parliament and of the Council, which forms the basis of food safety policy in the EU, also forms the basis of food imports to the EU. According to Article 11 of the said Regulation, in order for food to enter the EU market; it must meet EU standards or at least equivalent food standards (Anonymous 2). Rapid Alert System for Food and Feed (RASFF) is a system established by the European Union in 1979. The main purpose of the system is to ensure rapid exchange of information between member states for problems arising from risks that may arise in food and feed products, to take necessary measures and to protect consumers (Kugu et al., 2022). RASFF notifications are reports obtained as a result of the examination of samples taken from foods that are considered to pose a risk to food safety. Notifications can be of four different types: warning, information, border denial and news (Anonymous 3).

RASFF notifications are a common occurrence especially in Türkiye's fresh fruit and vegetable trade. This situation puts exports in trouble and may result in the destruction of the exported products.

Food Safety and Practices in Türkiye

With the increase in the diversity of food products, the fact that they go through many processes and stages from the field to the table raises questions about whether the food is healthy or not. Adulteration and imitation are very important in terms of food safety (Kantaroğlu & Demirbaş, 2019). Questions such as how the production is carried out, whether additives are added, whether health and sanitation rules are paid attention to, whether the production places are adequately inspected, whether the tools and equipment used are suitable for production are in the minds of conscious consumers (Sanchez et al. 2001). As a result of the development processes of countries, more attention has started to be paid to the content, reliability and healthiness of foods. As an example of these situations, traceability in the food sector is of great importance (Caswell et al. 2002).

In Türkiye, there have been some problems regarding food safety for many years.The main problems are; inadequacy of technical infrastructure from agriculture to processing industry, lack of technical personnel, inadequate training and awareness raising of producers and consumers, low purchasing power of consumers in general, weak use of technology (Erden, 2012).

Food legislation has been introduced to standardise these issues.Food legislation is basically the name given to the whole of the laws, by-laws and regulations issued to protect the consumer and determining the qualifications of the places where foodstuffs are produced, stored and sold in general terms. The first law on food safety in Türkiye is the Municipal Law No. 1580 enacted in 1930. Article 15 of this law listed the inspection of food production, storage and sales places within the municipal boundaries among the duties of the municipality. Paragraphs 2, 3, 28, 58 and 61 of these articles define the scope of this duty quite broadly (Giray and Soysal, 2007).

As noted by Barbaros et al. (2007), when fruit and vegetable exports are considered in general, Türkiye has a clear comparative advantage over other countries in raisins, dried figs, dried apricots, and hazelnuts. China, Türkiye and Brazil are the main suppliers of foodstuffs to the EU market. However, these countries should implement strict food safety regulations and measures based on RASFF notifications in order to gain a sustainable competitive advantage (Jaud et al., 2009). The excessive number of RASFF notifications for Türkiye, as in many developing countries in the food industry, may be due to insufficient resources and infrastructure (Çobanoğlu, 2013).

Contrary to popular belief, Türkiye is not completely self-sufficient in all agricultural products. While the EU is self-sufficient in cereals, meat and milk groups, it is found to be self-sufficient in fruit and vegetable groups In Türkiye, on the other hand, a trend in the opposite direction of the EU is observed; Türkiye is self-sufficient in fruit, vegetable and meat groups, while it is self-sufficient in cereals and milk groups (Niyaz and İnan, 2016).

The development of the global economy in the world and the fact that products are consumed in regions much different from the places where they are produced have revealed the necessity of more controlled monitoring of each stage of the product. With the developing technology, various computer-based software, interdisciplinary approaches and technological devices and new methods that facilitate the tracking of products have started to be used (Cebeci, 2006).

Although there are not many scientific studies on product verification and traceability systems in Türkiye, traceability services have started to be provided over the internet with the help of programmes established by certain organisations, user manuals have started to be published, courses and seminars have started to be organised and the demand for traceability systems has increased by large-scale companies (Çetin, 2014).

Türkiye's main and urgent problem in the field of food safety is the inability to record and control all components of food production practices. The lack of adequate control over the production, import and use of additives, which pose a high risk to public health and threaten public health if not used in appropriate doses, is a serious problem (Kantaroğlu and Demirbaş, 2019).

Briefly summarised, the most important problems in the field of food safety in Türkiye are traceability, deficiencies in the use of various technical knowledge and technology, problems in the implementation of the legislation and in this context, the excess of RASFF notifications in the export of products produced to the European Union can be shown as an example.

Mandarin Export Structure of Türkiye to the European Union and Other Countries

Citrus cultivation, which has a very old history in Türkiye, has developed rapidly after the Republic and has witnessed significant production increases until today. With its climate and soil structure, Türkiye is a country with extremely favourable ecological conditions for citrus cultivation. These citrus species are produced in the Black Sea, Aegean and Mediterranean regions of Türkiye (Oral, 2014).

Mandarin production in Türkiye by years is given in Table 1. Looking at the production, it can be predicted that there is an increasing trend from 1990 to 2022 and this production will increase based on the number of non-fruiting trees.

There is an increasing interest in European markets for mandarin, which is an early variety, and as a result, mandarin exports from Türkiye are gaining great importance and mandarin exports are of great importance in fresh fruit exports. Due to its favourable growing conditions and considerable export potential, it is of great importance for Türkiye to examine the production and marketing of this product (Can and Sulusoglu, 2019).

According to the data of 2022, mandarin is the most exported fresh fruit and vegetable product of Türkiye to the world countries. In this context, the total number of mandarin exports is given in Table 2.

Russia is an important trade partner in citrus exports. Russia ranks first in the list of the countries importing the most citrus fruits in the world, followed by France, Germany, the Netherlands and the UK (Atlı and Söyler, 2018). In this context, the price and quantity of mandarin exported by Türkiye to Russia are given in Table 3.

Table 1. Mandarin Production by Years (Source: TÜİK, 2023)

	Number of fruit-bearing trees	Number of non-fruit-bearing trees	Production (Tonnes)
1990	6.858.000	986.000	345.000
1995	7.825.000	793.000	453.000
2000	8.370.000	847.000	560.000
2005	9.230.000	1.347.000	715.000
2010	9.488.000	1.777.000	858.699
2015	11.786.000	2.230.000	1.156.365
2020	15.926.000	5.842.000	1.585.629
2022	19.620.000	5.054.000	1.865.000

Table 2. Quantity and value of mandarin exported from Türkiye to the world for the last five years (Source: Trademap, 2023)

2018	Production (Tonnes)	601.137
	Production Value ($)	241.202
2019	Production (Tonnes)	665.475
	Production Value ($)	297.951
2020	Production (Tonnes)	788.380
	Production Value ($)	390.285
2021	Production (Tonnes)	841.889
	Production Value ($)	399.948
2022	Production (Tonnes)	819.614
	Production Value ($)	418.667

Table 3. Mandarin exports of Türkiye to Russia (Source: TUİK, 2023).

	2018	2019	2020	2021	2022
Value (Thousand $)	119.859	163.833	219.576	235.210	274.393
Production (Tonnes)	245.308	305.460	417.819	438.679	467.759

Table 4. Main products most exported from Türkiye to the European Union (Source: Trademap, 2023)

Product	2020 production amount (tonnes)	2021 production amount (tonnes)	2022 production amount (tonnes)	Shares of the most exported products in 2022 (%)
Tomato	127.182	180.401	192.077	22.73
Hazelnut in shell	107.902	148.556	136.011	16.09
Lemon	92.925	104.201	116.197	13.75
Pepper	93.244	113.186	100.806	11.93
Watermelon	37.381	30.238	70.911	8.39
Grapefruit	81.698	59.364	58.441	6.92
Mandarin	53.829	49.665	50.865	6.02
Fresh or dry fig	46.353	48.584	45.741	5.41
Cucumber	32.372	46.493	39.127	4.63
Carrot and Radish	31.135	32.030	34.888	4.13
Total	704.021	812.718	845.064	100.00

Table 5. The most exported fresh fruit and vegetables from Türkiye to the European Union (Source: Trademap, 2023)

Product	2020 export value (thousand USD)	2021 export value (thousand USD)	2022 export value (thousand USD)	Percentage shares of export value of the top ten products in 2022 (%)
Fresh or dried hazelnuts	770.724	959.362	727.786	44.98
Tomato	109.563	158.371	210.634	13.02
Fresh or dry fig	164.975	190.602	155.105	9.59
Pepper	109.704	135.830	144.304	8.92
Shelled Pistachios	56.667	107.722	118.287	7.31
Lemon	67.094	64.843	79.085	4.89
Cherry	138.336	125.089	60.944	3.77
Various Tropical Fruits	45.262	54.446	52.332	3.23
Mandarin	35.138	33.331	36.973	2.29
Cucumber	28.278	41.074	32.584	2.01
Total	1.525.741	1.870.670	1.618.034	100.00

Table 6. Comparison of mandarin production and price with other most exported citrus fruits (Source: Trademap, 2023)

Ürün	Production (ton)	Export value (Bin $)	Share in citrus production (%)	Share in citrus export value (%)
Lemon	116.197	79.085	49.66	51.83
Grapefruit	58.441	31.797	24.98	20.84
Mandarin	50.865	36.973	21.74	24.23
Orange	8.465	4.726	3.62	3.10
Total	233.968	152.581	100.00	100.00

Dry and fresh fruit and vegetable exports to the Russian Federation are subject to various legislation. In this context, exports of fresh fruits and vegetables from Türkiye to the Russian Federation (RF) are carried out within the framework of the Memorandum of Understanding (Memorandum) which entered into force on 02 July 2008 and the Additional Memorandum signed on 09 April 2009 (Anonymous 4). These rules are mainly on standardisation and determination of pesticide residues. In the letter numbered E-21817801-305.04.02.02-5156332 of the General Directorate of Food Control under the Ministry of Agriculture and Forestry, it is stated that pesticide residue analysis should not be performed on every batch of products in the export of fresh fruits and vegetables to the Russian Federation and that the export of products can be allowed with the Safety Certificate, Form-1, Analysis Report and Phytosanitary Certificate for the appropriate batch according to the Russian Federation Maximum Residue Level list (Anonymous 5).

Mandarin ranks seventh in the list of the most exported fresh fruit and vegetable products of Türkiye to the European Union. The first in this list was tomato, the second was hazelnut and the third was lemon (Table 4).

Mandarin ranks ninth in the ranking of the export value of Türkiye's most exported fresh fruit and vegetable products to the European Union. In this list, hazelnuts ranked first, tomatoes second and figs third (Table 5).

In addition, when the exported citrus fruits are examined, it is seen that 50.865 tonnes of mandarin were exported according to the data of 2022. Mandarin is the second most exported citrus fruit according to 2022 data (Table 6).

Thomas Robert Malthus argued that due to the exponential growth of the human population, the fertility of the land would only increase arithmetically. As a result, resources would eventually become insufficient to support a constantly increasing population. Therefore, he believed that it may be necessary to consider restricting reproduction (Malthus, 1798; Karaca, 2022). At present, this argument cannot be applied. Although boosting production is crucial to meet the needs of the global population, it also gives rise to concerns regarding food safety. Although boosting production is crucial to meet the needs of the global population, it also gives rise to concerns regarding food safety.

This study analyses the food safety implications of Türkiye's mandarin exports to the European Union between 2019 and 2022 in the context of RASFF notifications. This study analyses the food safety implications of Türkiye's mandarin exports to the European Union between 2019 and 2022 in the context of RASFF notifications. The aim of this unique study is to develop strategies to address this issue in order to minimise export losses.

Material and Methods

In this study, the effects of food safety on mandarin trade between Türkiye and the European Union are analysed. In this context, the strengths, weaknesses, opportunities, and threats of this trade structure were determined and subjected to TOWS analysis by examining the literature reviews and RASFF notifications given by the European Union countries. SWOT analysis of internal strengths and weaknesses as well as external opportunities and threats is important for strategy formulation and development (Chang and Huang, 2006). TOWS analysis is a type of analysis based on associating threats and opportunities with weaknesses and strengths in the strategies to be formed (Weihrich 1982). The TOWS matrix is created, strengths and weaknesses, opportunities and threats are written on the matrix and strategies are created within this framework.

As a result of the analysis, strengths, weaknesses, opportunities and threats aspects of the mandarin export structure were identified. Based on these arguments, strategies were developed on the status of export and how to improve it and what the strategy can be.

Findings and Discussion

Türkiye has advantages such as geographical location, favourable climatic and environmental conditions, sufficient and diverse production amount, high yield in production areas. In addition to these advantages, there are serious problems in production, marketing, transport, storage and packaging. Problems arising from production and marketing prevent higher income from both domestic and foreign markets (Faryabi 2022).

Citrus exports in Türkiye first started in the 1950s but did not have a significant value until the 1970s. Since then, citrus exports to the former Eastern Bloc countries, which are currently the most important market for citrus fruits, have been an important step in this field. In this period, since both Türkiye and these countries did not have sufficient free foreign currency, trade was mostly carried out through the "Clearing" method (Güney, 2012). This is a slightly more developed form of barter. Importers in the countries signing the Clearing agreement pay the price of the goods they import in their national currencies to an institution such as the central bank or the Clearing office, which is assigned to keep the price of the goods they import in Clearing accounts in their own countries. The accounts are balanced at the end of the period with the accounts consisting of the money deposited by the importers of the other country to their own relevant institution. If there is a deficit, this deficit is closed with any convertible foreign currency agreed in advance (Küsmez, 2016).

One of the most important items in the trade relationship between Türkiye and the European Union is the fresh fruit and vegetable trade. In this context, some problems can be seen in fresh fruit and vegetable trade. Gürbüzer (2008) showed that the current problems in the export of fresh fruit and vegetable production are transportation, protectionism in the importing country, difference in product standards, difficulties in product promotion, bureaucratic problems in Türkiye, communication with the importing company, inspection problems, problems at customs, bureaucratic obstacles and lack of market analysis. In addition, in the same study, HACCP and GLOBALGAP standards, which are related to product safety and traceability of products, are among the most important issues sought by importing countries.

The problems experienced by the Turkish citrus export sector can be analysed in two aspects as internal and external problems. Intrinsic problems are the lack of stable markets with reliable importers, the lack of effective intermediaries or institutions, the inability to create product diversification, and the low level of technology use, so that it cannot benefit from the cost advantages that can be obtained with technology. External problems include high costs of transport and packaging, inappropriate quality standards and payment failures, especially in Ukraine, Russia and some Eastern European importers (Zenginoğlu and Djik, 2006). Various policies are applied in the citrus trade relationship with the European Union, Türkiye's other major trading partner. These policies can be classified as product standards, producer organisations and branch associations, enterprise funds and programmes, intervention regulations and recalls. In some of the comments and opinions on the comparison of the Turkish citrus sector with the EU citrus sector, it is stated that the amount of refunds applied for citrus exports is too high. However, although these refund amounts are low, it can be stated that the aids and supports applied by the EU in the citrus sector from production to export are made at every stage and in a wide variety of directions and that these supports have a great share in the visible positive results (Zenginoğlu, 2007).

Table 7. TOWS Matrix (Source: Weihrich, 1982)

	Weaknesses	Strengths
Threats	Weaknesses – Threats (W – T)	Strengths – Threat (S – T)
Opportunities	Weaknesses – Opportunities (W – O)	Strengths – Opportunities (S – O)

In this context, orange and mandarin exports are carried out within the scope of the European Union Commission Implementing Regulation 2019/1793 and the Model Certificate Issuance Procedure for Fresh Fruit and Vegetable Exports to European Union Countries (Anonymous 6). In addition to these, mandarin trade with the United Kingdom is carried out with the criteria determined by the Food Standards Agency based on European Union practices. According to these criteria, some pesticides known to be harmful to human health are banned and their residues cannot be found in the products. The measure of the amount of heredity in the product is expressed as MRL (Maximum Residue Level). The amount of residue can only be found within the limits permitted by the European Commission (Anonymous 7, Anonymous 8). Products exceeding these limits are either destroyed or returned.

In this framework, when the Rapid Alert System for Food and Feed (RASFF) notifications given by the member countries are analysed, it is seen that the chemicals that are banned according to the regulations and criteria and have the most residue problems are prochloraz, fembutatin oxide, esfenvalerate, chlorpyrifos methyl (Anonymous 8). Although these active substances are authorised in the Plant Protection Database, their use in mandarin may cause problems in exports. The first of these problems is whether these active ingredients are authorised for mandarin or not. It is forbidden to use pesticides that are not licensed for mandarin. In addition, the pesticides licensed for mandarin are also evaluated within certain residue values.

Apart from residues, aflatoxins, the other undesirable substance subject to RASFF notifications, are included in mycotoxins in taxonomy. They are mycotoxins that can be widely found in many foods including cereals, oilseeds, spices, meats, milk and dairy products and animal feeds. Foods and animal feeds can be contaminated with aflatoxins during product processing, storage and sale. Aflatoxin contamination levels may vary according to climatic, regional characteristics or food type (Yentür and Er, 2012). Consumption of aflatoxin-contaminated feeds adversely affects animal health and production. At the same time, consumption of meat, eggs and milk of these animals poses a danger to human health (Gowda et al. 2004).

RASFF notifications are published periodically every month by the European Union. These notifications are open to everyone. Thus, the member countries of the Union can see how many RASFF notifications have been received for the products they import from which country. This may indicate that countries can organise their own food safety policies according to the notifications from here. In this way, they can inspect the products they follow where they come from according to the notification results.

Table 7 shows the list of RASFF notifications made for all dry and fresh fruit and vegetable products coming from European Union countries by years. It can be said that the notified products were withdrawn from the market, recalled or destroyed or returned. Here, it can be concluded that "the company's request is taken into consideration and the goods are sent back for reprocessing if the company wishes to do so". If this is not possible, the products are destroyed by the competent authorities. The returned products can also be re-entered to Türkiye after the necessary controls in accordance with the Law No. 5996 on Veterinary Services, Plant Health, Food and Feed of the Ministry of Agriculture and Forestry. Products in violation of this law are destroyed (Anonymous 9).

In the table, the high number of RASFF notifications from Bulgaria and Germany is noteworthy. The reason for this is that trucks loaded with products exported from Türkiye generally use the Bulgarian border while leaving the country. Kapıkule Border Gate has been one of the most important gates of Turkish foreign trade to Europe since the past years (Küçükaltan 2012). In addition, the large Turkish population in Germany also explains that Turkish products are very popular in the country and food trade with Türkiye may be high. In this context, it is considered that the increase in the number of RASFF notifications in 2021 is a result of increased food safety inspections due to the pandemic in the Bulgarian border and in Germany. Especially in Germany, market and company controls are carried out intensively. In the controls on aflatoxin and residues, products are recalled or destroyed.

Apart from this, inspections at the Greek border and RASFF notifications from there are also noteworthy. Although the Bulgarian border is mainly used for fresh fruit and vegetable exports, the Greek border also serves as an important gate for exports. In this context, especially dried fruit exports and other food products can be inspected from here. Therefore, RASFF notifications from Greece are mainly generated as a result of border controls.

In the notifications made by the European Union, aflatoxin content has been reported in products such as dried figs, dried grapes and dried apricots. Products contaminated with aflatoxin are detained and destroyed by the relevant authorities. When the RASFF notifications were analysed, it was determined that aflatoxin was predominantly found in the notifications made, especially in dried products, except for high MRL values or residues. In addition to dried fruit and vegetables, aflatoxin is a serious problem for walnuts, hazelnuts, pistachios, sesame seeds and processed products (halva, tahini, etc.). Especially in countries such as France, the Netherlands, Italy, Belgium and Switzerland, aflatoxin can be found in products during field inspections and in warehouse controls based on consumer notifications. In this context, it can be said that a significant portion of the RASFF notifications received outside Bulgaria and Greece, where active ingredient analyses are performed at the border, are based on aflatoxins. As a result of the fact that these notifications are open to all European Union countries, countries want to remove aflatoxins and other mycotoxins found in imported foods by tightening inspections on products entering through their borders. According to Trademap data, this is an important problem for Türkiye, which is the world's largest hazelnut and dried fruit exporter country (Anonymous 10).

It can be said that countries such as Romania, Slovenia, Sweden and Denmark have recently increased their field inspections in line with their own food safety policies by following the current RASFF notifications. In this context, products with residue values above the permitted MRL values and aflatoxin-formed products are collected from the market and the return or destruction procedure is applied.

Table 8. 2019 – 2022 RASFF Notifications (Source: Anonymous 11)

2019				2020			
C	TNN	MN	PMN	C	TNN	MN	PMN
France	28			France	21		
Italy	34			Italy	11		
Bulgaria	84	5	5.95%	Bulgaria	149	22	14.77%
Norway	1			Norway	3		
Cyprus	1			Lüksemburg	1		
England	25			England	9		
Germany	79			Germany	61		
Poland	3			Poland	5		
Netherlands	15			Netherlands	15		
Slovakia	2			Slovakia	6	2	33.33%
Spain	3			Spain	7		
Belgium	7			Belgium	9		
Czechia	3			Czechia	2	1	50.00%
Denmark	6			Denmark	6		
Sweden	5			Sweden	7		
Finland	1			Finland	1		
Croatia	3			United Kingdom	8		
Austria	3			Austria	4		
Switzerland	2			Switzerland	7		
Greece	8			Greece	1		
Romania	2			Romania	6		
Lithuania	1			Lithuania	1		
Malta	1			Arnavutluk	2		
Hungary	1			Hungary	3		
Slovenia	7			Slovenia	3		
Estonia	0			Estonia	0		
Portugal	0			Portugal	0		
Ireland	0			Ireland	0		
Latvia	0			Latvia	0		
Total	325	5	1.54%	Total	348	25	7.18%
2021				2022			
France	16			France	25		
Italya	29			Italya	37		
Bulgaria	258	80	31.01%	Bulgaria	223	20	8.97%
Norway	4	1		Norway	2		
Cyprus	1			Cyprus	0		
England	0			England	0		
Germany	106	1	0.94%	Germany	71		
Poland	9	1	11.11%	Poland	16	2	12.50%
Netherlands	14			Netherlands	25		
Slovakia	3			Slovakia	6	2	33.33%
Spain	6			Spain	7		
Belgium	12			Belgium	18		
Czechia	0			Czechia	1		
Denmark	9			Denmark	3		
Sweden	3			Sweden	6		
Finland	2			Finland	2		
Croatia	12	2	16.67%	Croatia	5		
Austria	10	1	10.00%	Austria	9		
Switzerland	4			Switzerland	7		
Greece	9			Greece	16		
Romania	22	1	4.55%	Romania	22		
Lithuania	0			Lithuania	1		
Malta	1			Malta	1		
Hungary	3			Hungary	1		
Slovenia	15	2	13.33%	Slovenia	7		
Estonia	7			Estonia	0		
Portugal	0			Portugal	2		
Ireland	1			Ireland	2		
Latvia	5	1	20.00%	Latvia	3	1	33.33%
Total	561	90	16.04%	Total	518	25	4.83%

C: Country; TNN: Total Number of Notifications; MN: Mandarin Notifications; PMN: Percentage share of mandarin notifications compared to all notifications (%)

Table 9. Number of RASFF notifications by years and Türkiye's situation (Source: Anonymous 11)

	Total RASFF Notifications	RASFF Notifications Related to Mandarin Ratio (%)	Ratio (%)
2019	325	5	1.54
2020	347	25	7.20
2021	561	90	16.04
2022	518	25	4.83

Table 10. TOWS matrix and analysis

Weaknesses	Strengths
• Poor traceability • Russian Federation - as the main market • Unconscious producer - engineer • Not knowing systems such as GLOBALGAP, HACCP • Inadequate residue controls • Lack of standardisation • Non-compliance of production with EU legislation • Failure to adopt modern production techniques • Inadequacies in storage and the impact on supply	• Geopolitical position • Experiencing the four seasons • The region's abundance of arable land ensures abundant production. • Transport facilities
Threats	
• Failure to keep traceability records • Russian Federation, the largest trading partner of the Türkiye, may view trade with the EU negatively due to political situations. • Yield losses due to failure to switch to modern production • Low value of the product due to problems in standardisation • Excessive number of RASFF notifications and therefore spoilage of products not received due to residues • Excessive proliferation of unwanted pests	
Weaknesses – Threats (W – T)	**Strengths – Threats (S – T)**
• Necessary measures should be taken to ensure correct traceability • An alternative to the Russian market should be created by developing trade with the European Union countries. • Elimination of inadequacies in mandarin storage and restructuring of warehousing activities in accordance with EU legislation. • Producer and engineer awareness should be raised and modern production techniques should be made widespread, yield should be increased, and the proliferation of unwanted pests should be prevented • Residue controls should be increased and carried out correctly within the legislation. The number of RASFF notifications should be reduced. • Standards should be brought in line with EU norms and studies should be carried out on this subject • GLOBALGAP - HACCP standards should be generalised. Production must comply with EU legislation and be an example. • Importance should be given to storage activities against aflatoxin production	• Despite its geopolitical location and ease of transport, this advantage should be prevented from not being utilised in marketing. • Modern production and storage facilities to take advantage of four-season production, with storage conditions in line with EU negotiations. • Abundant production, coupled with adherence to the EU agricultural policy, has resulted in a significant increase in product prices. • Reducing RASFF notifications and controlling pest reproduction will significantly improve production and transportation.
Opportunities	
• Proximity to everywhere thanks to the location and the possibility to develop the market • Continuity in production under favourable conditions • Increased exports due to abundant production • - Development of production through land, sea and air routes	
Weaknesses – Opportunities (W – O)	**Strengths –Oppotunities (S – O)**
• Poor traceability hinders EU exports. • Although production is continuous under favourable conditions due to unconscious producer - engineer, the value of the product produced decreases. • Despite the high level of transport facilities, problems arise due to food safety. This situation prevents the utilisation of this advantage.	• Geopolitical and geographical location is a very important advantage in exporting to the EU. It provides convenience in export. • The suitability of the seasons appears as continuity in mandarin production. Every season eliminates supply supply problems and increases earnings • The development of land, sea and air routes enables the product to be shipped fresher.

When RASFF notifications are analysed by year, it is seen that there was a significant jump in 2021. In 2021, there were 90 RASFF notifications related to mandarin, all of which were related to residues and mycotoxin (aflatoxin). This number corresponds to 16,04% of the total RASFF notifications in 2021.

As can be seen from the figure, there is a serious jump in 2021. This jump is considered to be due to increased food safety inspections due to the COVID - 19 pandemic. In addition, the increase in the country's export potential in recent years and more products being sent to the European Union may have increased the number of notifications in direct proportion. In 2022, the number of notifications decreased. In this regard, the effect of the calls of exporters' associations, awareness-raising activities and tightening of controls throughout the country can be mentioned.

The TOWS analysis created in this context is given in Table 8. According to this table, which shows the Dry and Fresh Fruit and Vegetable Trade between the European Union and Türkiye in plain terms, it is essential to formulate some strategies.

In the light of the TOWS analysis given above, the strengths of Türkiye's mandarin exports to the European Union are as follows.

- Türkiye's geopolitical position and its proximity to the European Union countries
- Four seasons in the country and the possibility of continuous production
- Ease of transport of the produced product
- The region's abundance of arable land ensures abundant production.
- In the light of the same analysis, weaknesses are given as follows.
- Weakness of traceability activities
- Russian Federation – as main sales market
- Lack of knowledge of producers and people guiding producers
- Systems such as HACCP, Global GAP are not widespread
- Inadequate residue controls
- Lack of standardisation
- Problems in the application of modern agricultural techniques
- Storage and packaging problems. Aflatoxin formation.

Threats are given as follows;

- Failure to keep traceability records
- Russian Federation, the largest trading partner of the Türkiye, may view trade with the EU negatively due to political situations.
- Yield losses due to failure to switch to modern production.
- Low value of the product due to problems in standardization.
- Excessive number of RASFF notifications and therefore spoilage of products not received due to residues.
- Excessive proliferation of unwanted pests.
- The aspects that can be seen as opportunities in the light of TOWS analysis are as follows.
- Proximity to everywhere thanks to the location and the possibility of market development
- Continuity in production under favourable conditions
- Increased exports due to abundant production
- Development of production through land, sea and air routes.

Conclusions and Recommendations

In this study, the effect of food safety criteria on Türkiye's dry and fresh fruit and vegetable exports to the European Union is analysed on the basis of mandarin sample. In this context, the concept of food safety has been analysed and a literature review has been conducted. On the axis of RASFF notifications made by the European Union, the trade of fruit and vegetable with the European Union has been analysed through food safety. In the light of the literature and legislation reviews, findings have been put forward and these findings have been subjected to TOWS analysis. After the strengths, weaknesses, opportunities and threats of the subject were revealed, they were matched and compared with each other. According to these data;

- Necessary measures should be taken to ensure that traceability activities are carried out correctly and appropriately. Traceability is a very important issue in European Union legislation. For this reason, recording procedures should be implemented meticulously.
- Despite the geopolitical location and the favourable climatic conditions for the production of mandarins, these advantages cannot be exploited. Therefore, the deficiencies in the storage of mandarins should be eliminated. Storage and production should be brought into line with European Union regulations.
- Producers and those who guide them should be made aware. Unconscious production leads to problems in food safety and standardisation. This leads to loss of value and other losses in the products produced. For this reason, producers and those who guide them should be raised awareness. Especially in exports to the European Union and the United Kingdom, it is essential to know the MRL (Residue) values and prohibited active substances and to comply with these values.
- It is important to use modern agricultural techniques in the production of mandarins. In the production of mandarins, it is important to select varieties appropriate to the region, to use the necessary amount of inputs in production and to avoid overuse, and to carry out production activities appropriate to the variety and the region. As technology changes, the aim should be to increase yield and quality with less input. For this reason, it is essential for producers to keep up with the changing world by adopting new pruning techniques and using more appropriate fertilisers and pesticides for the product.
- It is also essential to establish a traceability mechanism by recording these processes. Traceability is the recording of all stages of production in the light of the principle of traceability from field to fork. in this way, errors occurring during production are minimised. full transparency is ensured. It is an

indispensable condition for mandarin trade with the EU. Therefore, it is essential for Türkiye to develop agricultural traceability activities.

- Unnecessary spraying and fertilisation in fruits and vegetables should be avoided in the light of the principle of from field to table. Producers should be made aware of this issue and production, especially in export areas, should be carried out under the control of expert engineers.
- Residue controls should be increased in accordance with the procedures. Products that cannot enter due to residue are subjected to treatments such as destruction or return. This situation causes problems in the value or shelf life of the product.
- Especially in dried fruits and vegetables, the aflatoxin problem is serious. In this context, importance should be given to the packaging and storage of products. In order to prevent the formation of aflatoxin, the legislation on packaging, storage and public supply established for the products must be strictly complied with.
- GLOBALGAP and HACCP standards should be disseminated and production should be compatible with European Union legislation. Compliance with these standards can also contribute to residue and standardisation problems. This may increase labour costs, but this is negligible compared to the increase in selling price and profitability due to the cleaner product.

In the summary of the analyses, it was observed that traceability and residue problems were quite high in vegetable and fruit exports to the European Union, especially mandarins. In this framework, it can be said that producers and those who direct them are unconscious about food safety, and the high number of RASFF notifications is a result of not paying attention to food safety. It is important to raise awareness of the producers that they should wait for the period between harvest and pesticide application and pay attention to storage conditions.

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Effect of Maltodextrin Concentrations and Drying Temperature on the Physico-chemical Characteristics and Color Measurements of Butterfly Pea Flowers *(Clitoria ternatea* L*)* Powder

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ABSTRACT

Butterfly pea flower (*Clitoria ternatea* L.) is a tropical plant that is rich in bioactive compounds, especially anthocyanins which are useful as natural dyes and antioxidant compounds. The bioactive compounds of butterfly pea flowers are unstable due to environmental influences, especially temperature, oxygen, light and acidity. In order to improve the stability of bioactive compounds, especially anthocyanin compounds in powder form, it is necessary to utilize encapsulation technology using coating materials. The aim of this research was to determine the effect of maltodextrin concentration and drying temperature on the physico-chemical characteristics and color measurements of encapsulated butterfly pea flower extract. The research method used was a factorial design prepared using a randomized block design consisting of 2 factors. Factor I (maltodextrin concentration) consisted of 3 levels, namely (10%, 20%, and 30%) while factor II (drying temperature) consisted of 3 levels (70ºC, 80ºC, and 90ºC), with 3 repetitions. The observation variables are: a) antioxidant activity, b) anthocyanin content, c) water content, d) dissolution time, e) color properties (L^*, a^*, and b^*). Based on general research results, a maltodextrin concentration of 10% and a drying temperature of 70ºC showed the best results based on antioxidant activity rate and the highest anthocyanin content (51.47% and 47.36mg/g), as well as color measurements with the lowest L^* value = 52, highest a^* value = +2.6, and highest b^* = -11.16. Except for powder solubility, a maltodextrin concentration of 30% and a drying temperature of 90ºC resulted in the fastest solubility time (16.67 seconds). For water content, all treatments were still in accordance with spice standards in Indonesia and standards issued by the USDA.

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Introduction

Butterfly pea flower (*Clitoria ternatea* L) is a plant that is rich in antioxidant compounds, especially anthocyanins. Anthocyanins are a group of flavonoid compounds which are also used as natural dyes, namely to produce the orange, red and purple colors commonly found in many flowers and fruits. Reactions that occur due to the presence of light, metal, high temperature, and high pH generally result in the destruction of anthocyanins. Provenzi et al. (2006), in Vanini et al. (2009), stated that the stability of anthocyanins was influenced by pH, temperature, oxygen and light. According to Reyes and Zevallos (2007), the color deterioration of anthocyanin pigments is caused by the change of the red flavilium cation into a colorless carbinol base and finally into colorless chalcone.

Increasing temperature will have the impact of increased anthocyanin oxidation, which will cause anthocyanin degradation. According to Jian Hie (2004), when the chalcone compound becomes unstable at high temperatures, it then degrades into a brown compound. Another possibility is that at high temperatures it will result in hydrolysis of the 3-glucose structure so that the anthocyanin changes into the unstable anthocyanidin.

Instant powder is a semi-finished food product in the form of powder or fine granules made from spices, seeds, fruits or flowers, and is usually served quickly by brewing wherein it is dissolved in cold or hot water. According to Ramadhia (2013), the advantages of instant powder are that it is practical, has extended shelf life due to the low water content, and its smaller volume makes packaging and distribution easier. The characteristics of instant powder are that it has the same color, smell, taste, and appearance

as fresh products, and has good nutritional characteristics and storage stability (Permata and Sayuti, 2016).

Making instant powder, especially from liquid materials such as anthocyanin extract, requires a coating/encapsulating material, while the process is known as encapsulation. Coating ingredients are ingredients added during the food processing process to coat flavor components, increase the total amount of solids, increase volume, speed up the drying process, and prevent damage to ingredients due to heat. In this research, maltodextrin coating material was used.

Maltodextrin is an imperfect starch hydrolysis compound, consisting of a mixture of sugars in simple form (monosaccharides and disaccharides) in small quantities, short-chain oligosaccharides in high quantities, and long-chain oligosaccharides in small quantities (Hadnađev et al., 2011). Maltodextrin is often used as a filler in encapsulation because it has good coating properties due to its ability to form emulsions and its low viscosity (Khrisnan, et al., 2005). Apart from that, maltodextrin is widely used because it is technically easy to find (Moore et al., 2005). Maltodextrin can undergo rapid dispersion, has a high solubility, can form a matrix, has a low chance of browning, can inhibit crystallization, has strong binding capacity, and has low viscosity compared to starch (Supriyadi and Sakha, 2013). Maltodextrin is a water-soluble substance and can protect the encapsulated substance from oxidation reactions (Septevani, et al., 2013). Maltodextrin can also reduce agglomeration problems during storage so that it can increase product stability (Gabas, et al., 2007). Hairunnisya (2016) reported that the differences between maltodextrin and starch included the sweeter taste of maltodextrin and faster absorption, which was because maltodextrin had a simpler polymer form. But when compared with simple sugars (dextrose, fructose and sucrose), the absorption time of maltodextrin moved more slowly. According to Yongki (2008), maltodextrin is defined as a starch hydrolysis product containing α-D-glucose units which are mostly bound through 1,4 glycosidic bonds with a DE of less than 20. Maltodextrin can be used in food because of advantages such as being able to pass through the dispersion process fast, has high solubility, can form films, has low hygroscopic properties, and can inhibit crystallization (Ekpong et al., 2016). Maltodextrin can be used in the encapsulation process to protect anthocyanin compounds and to protect compounds that are sensitive to oxidation or heat (Silitonga and Sitorus, 2014). Maltodextrin is a water-soluble material, which when used as a packaging material can protect the encapsulated active substance from oxidation reactions (Ersus and Yurdagel, 2007). The process of encapsulating the active substance with packaging material can protect the active substance from external factors and increase the stability of the active ingredient so that its functions can be maintained during storage. Maltodextrin can also reduce agglomeration problems during storage thereby increasing product stability (Gabas et al., 2007). Pratiwi (2011) stated that maltodextrin is a filler commonly used to form the body in the making of powdered drinks.

Food drying has the aim of being a means of extending shelf life by reducing the water content to prevent the growth of spoilage microorganisms and minimize food distribution costs, because the weight and size of the food are lower (Wicaksono, 2012). Martunis (2012) stated that the drying time and temperature used couldn't be determined with certainty for each food ingredient, but depended on the type of material being dried, such as the type of powdered food ingredient using an oven with varying drying temperatures of 60ºC to 90ºC for 5 to 7 hours.

As a coating material, maltodextrin has several advantages, namely: easy to dissolve, reduces agglomeration during storage, low viscosity, easy to find, and cheaper. However, research that specifically explores the concentration of maltodextrin coating material with oven drying temperature for encapsulating butterfly pea flower extract is still too few, even if it is used for coating watermelon extract, and most of the research topics found are about the use of types of coating materials and drying methods, both individually and in combination. The aim of this research was to determine the effect of maltodextrin concentrations and drying temperature on the physico-chemical characteristics and color measurements of encapsulated butterfly pea flower extract

Materials and Methods

Sample Preparation

The butterfly pea flower samples were collected from Mulyo Santoso's Garden, Sukun District, Malang City, East Java, Indonesia. The planting location is in full sunlight with a planting medium mixed with soil, sand and manure. Fertilization uses organic fertilizer and agricultural lime which is given once every 2 weeks.The butterfly pea flowers were picked when in bloom, then while still fresh and separated from the stems, were sorted. Only those that were still intact were selected. The samples were then reduced in size by chopping them with a stainless-steel knife.

Extraction

After reducing the size of the butterfly pea flowers, the extraction process was carried out using the maceration (soaking) method. Soaking was done using boiling water as a solvent in a ratio of 1:8 (1 g butterfly pea flowers: 8 ml water). modified based on research by Yudiono (2011). The solution was stirred for 5 minutes and afterwards was filtered using filter paper, to obtain the butterfly pea flower filtrate.

Powder Production

The filtered butterfly pea flower filtrate was added with maltodextrin at levels of 10%, 20%, 30% of the filtrate. The solution was stirred again until it became homogeneous using a magnetic stirrer to ensure that the solution was evenly mixed. Then it was dried using a drying oven (three ovens available) at the temperatures of 70ºC (first oven), 80ºC (second oven), and 90ºC (third oven) with the same drying time of 7 hours. This drying process followed the research procedures of Yogaswara et al. (2017), namely the modified thin layer drying. The final stage was the flouring process using a blender, and in order to get the same size shape, sieving was carried out with a size of 60 mesh (mm add).

Determination of Antioxidant Activity (Jabbar et al. 2019)

4 ml of the sample solution was taken, then 1 ml of DPPH solution was added with a concentration of 0.2 mM, then the solution was left for 30 minutes before analysis. Afterwards, 1 ml of the solution was taken and the absorbance was measured at a wavelength of 517 nm.

$$\text{E-DPPH} = \left[\left(\frac{A0 \ -A1}{A_0}\right) \times 1000\right] \quad (1)$$

Here;

E-DPPH= Effect of DPPH capture (%)

A0 = absorbance of the control or without the addition of DPPH

A1 = absorbance of the sample

Determination of Anthocyanin Levels (Lee et al., 2005)

About 1g of anthocyanin powder was placed in two different test tubes and diluted. The first test tube was added with 10ml of KCl (pH 1.0) buffer solution and the second test tube was added with 10ml of CH_3CO_2Na (pH 4.5) buffer solution. Using a spectrophotometer, the absorbance values of the sample were determined at 520 nm and 700 nm wavelength. The absorbance value of the sample was calculated by using (Eq 2): (Giusti and Wrolstad, 2003)

$$\text{Absorbance (A)} = [(A_{520\,nm} - A_{700\,nm})\ pH\ 1.0 - (A_{520\,nm} - A_{700\,nm})\ pH\ 4.5] \ldots\ldots\ldots\ldots \quad (2)$$

The total anthocyanin in the sample was calculated as cyanidin-3-glucoside following (Eq. 3):

$$\text{Total Anthocyanin (mg/L)} = \frac{A\ x\ MW \times DF\ X\ 10^3}{\varepsilon\ x\ l} \quad (3)$$

Here;

A = (A520nm- A700nm) pH1.0 - (A520nm- A700nm) pH4.5

MW = Molecular Weight (448.8 g/mol forcyanidin-3-glucoside)

DF = Dilution Factor

103 = Conversion from gram to milligram

ε = Molar extinction coefficient, L × mol–1 × cm–1 (26,900 L/mol/cm for cyanidin-3-glucoside)

l = Pathlength (1 cm)

Determination of Water Content (AOAC, 2005)

Before using the cup, it was first baked in the oven for 30 minutes at a temperature of 100-105ºC. Then the crucible was cooled in a desiccator to remove any water vapor and weighed (A). The sample was weighed as much as 2 g in a dry cup (B), then it was baked in an oven for 6 hours at the temperature of 100-105ºC. The sample was next cooled in a desiccator for 30 minutes and weighed (C). This stage was repeated until a constant weight was achieved. Determination of water content was calculated using the formula:

$$\text{Water content (\%)} = \frac{B-C}{B-A} \times 100\% \quad (4)$$

Here:

A: weight of empty cup (g)

B: cup weight + initial sample (g)

C: cup weight + dry sample (g)

Dissolving Time Test (Andhika, 2016)

The sample was weighed at 5 grams and dissolved in 50 ml of water, then stirred until homogeneous using a magnetic stirrer and a light, to determine whether the powder dissolved in water contained sediments or not. In this study, solubility was calculated based on the time the butterfly pea flower powder dissolved completely in seconds (s).

Color Properties (Colorimeter AMTAST AMT506)

Color test measurements used the AMTAST AMT506 Colorimeter. The parameters measured include the L^* (lightness) value, which means it tended to have white, gray, and black achromatic colors; the a^* value represented the color red (+) to green (-) and the b^* value represented the color yellow (+) to blue (-). The steps of using this tool are as follows:

- Turn on the colorimeter by moving the power switch button to the 'on' position.
- Select color space L, a^*, b^* by pressing the Lab button.
- Place the tip (focus lens) of the tool on the sample target which had been placed in the plastic above
- Press the measuring button.

Measurement of the target color on the sample was completed when the indicator beeped and the target color results appeared on the display.

Results and Discussion

Antioxidant Activity

The average values of antioxidant activity of instant butterfly pea flower powder with the addition of varying levels of maltodextrin and drying temperatures are presented in Table 1.

Table 1 shows that the average value of the antioxidant activity of instant butterfly pea flower powder ranges from 6.3% to 51.47%. The highest average value of antioxidant activity is found in the treatment of adding variation in maltodextrin content of 10% and a drying temperature of 70°C, which is 51.47%. While the lowest average value of antioxidant activity is found in the treatment of adding variation in maltodextrin of 30% and a drying temperature of 90° C, which is 6.3%.

The increase of maltodextrin concentration and drying temperature can cause a decrease in the antioxidant activity of the instant butterfly pea flower powder. This is because the greater the amount of the total solids contained in the material, namely maltodextrin as a filler, the smaller the measured antioxidant activity became. Alternatively, it can also be caused by changes in antioxidant compounds as a result of the drying process using high temperatures, which causes the phenolic compounds to decompose so that their ability as antioxidants decreases (Estiasih, 2009). Heat can cause the decomposition of antioxidant compounds into other forms, resulting in a decrease in antioxidant activity, and bioactive components such as flavonoids and phenols being damaged at temperatures above 50 °C. The results of the antioxidant activity test showed that the combination of temperature and maltodextrin concentration had an effect on the stability of antioxidant compounds, where the maltodextrin concentration of 20% in various temperature

treatments appeared to be the most stable compared to other treatments. Temperature treatment causes instability of antioxidant compounds (the higher the drying temperature, the worse the impact) while maltodextrin plays a role in protecting the stability of these compounds from external factors (drying temperature), but in this study a concentration of 20% provided the best protective effect. Fathinatullabibah et al. (2014) confirmed that flavonoids were unstable at 70ºC. It was further revealed that flavonoids were a class of compounds that were not heat resistant and were easily oxidized at high temperatures (Rompas et al., 2012)

Anthocyanin Levels

The average values of anthocyanin content of instant butterfly pea flower powder treated with variations in maltodextrin and drying temperatures are presented in Table 2.

Table 2 shows that the average value of the anthocyanin content of instant butterfly pea flower powder ranges from 19.02 – to 47.36 mg/g. The highest average value of anthocyanin content is found in the treatment of adding variations in maltodextrin content of 10% and a drying temperature of 70°C, which is 47.36 mg/g. While the lowest average value of anthocyanin content is in the treatment of adding variation in maltodextrin content of 30% and drying temperature of 80°C, which is 19.02 mg/g.

Increasing the concentration of maltodextrin and drying temperature can cause a decrease in anthocyanin levels in instant butterfly pea flower powder. This is because the addition of higher levels of maltodextrin will increase the solidity of the material so that the amount of anthocyanin levels decreases. Related to this, maltodextrin is a polysaccharide. With heat treatment, simpler compounds are formed, both disaccharides (sucrose) and monosaccharides (glucose). The formation of monosaccharides and disaccharides at high temperatures will cause a brown compound (melanoidin) through the Maillard reaction or caramelization. The formation of this brown color has the effect of decreasing the absorbance value so that the higher the maltodextrin the anthocyanin test results are also lower. According to (Cao et al., 2009; Rein, 2005) heating causes the degradation of sugar into furfural and 5-hydroxymethyl-furfural and reacts with anthocyanins to form brown products. This is in accordance with previous research results (Marsin et al., 2020; Hariadi et al., 2018; and Ariani, 2005). Similar results occurred in research by Padzil et al. (2018), in which increasing the concentration of maltodextrin would reduce the total monomeric anthocyanin content of purple sweet potato extract. Increasing the drying temperature causes the anthocyanin content obtained to be smaller because at high temperatures the anthocyanin degrades into ketone products. According to Dai (2010), at temperatures of more than 70°C the degradation of anthocyanins will be quite significant. Damages due to drying can occur in two stages, namely hydrolysis of the anthocyanin glycosidic bonds, resulting in unstable aglycones, and the aglycone rings open to form carbinol and chalcone groups which will cause color changes (Jian He, 2004). At the 30% maltodextrin treatment, the drying temperature was increased to 90°C, but the anthocyanin content did not differ/had no effect. This shows that the higher the concentration of the encapsulate/coating material, the stronger the protective power of the core material (anthocyanin) against heat treatment. The structure of the maltodextrin molecule is spiral-shaped so that the flavor molecules as the core ingredient will be trapped in the spiral helix structure. Thus, the addition of maltodextrin will be able to reduce the loss of chemical components during the heating process (Gustavo, et al., 1999)

Water Content

The average values of the water content of instant butterfly pea flower powder with the addition of variations in maltodextrin content and drying temperatures are presented in Table 3.

Table 1. Average values of antioxidant activity (%) instant butterfly pea flower powder

Maltodextrin Concentrations	Drying Temperatures		
	70°C	80°C	90°C
10%	51.47g ± 0.29	41.98f ± 0.19	36.2de ± 4.11
20%	38.42e ± 1.92	34.48d ± 0.90	30.12c ± 1.85
30%	28.75c ± 0.29	10.69b ± 1.06	6.3a ± 0.50

Note: Values followed by different letters indicate significantly different results in the Duncan test (α=5%)

Table 2. Average values of anthocyanin content (mg/g) instant butterfly pea flower powder

Maltodextrin Concentrations	Drying Temperatures		
	70°C	80°C	90°C
10%	47.36f ± 0.16	43.04e ± 1.19	29.69c ± 0.28
20%	31.61d ± 0.43	24.02b ± 2.41	23.06b ± 0.29
30%	19.89a ± 0.28	19.02a ± 0.29	20.27a ± 0.16

Note: Values followed by different letters indicate significantly different results in the Duncan test (α=5%)

Table 3. Average values of water content (%) of instant butterfly pea flower powder

Maltodextrin Concentrations	Drying Temperatures		
	70°C	80°C	90°C
10%	6.75g ± 0.17	6.14f ± 0.22	4.72c ± 0.32
20%	6.51g ± 0.13	5.17d ± 0.25	3.65b ± 0.14
30%	5.73e ± 0.16	4.36c ± 0.18	2.99a ± 0.23

Note: Values followed by different letters indicate significantly different results in the Duncan test (α=5%)

Table 4. Average values of dissolution time (s) of butterfly pea flower instant powder

Maltodextrin Concentrations	Drying Temperatures		
	70°C	80°C	90°C
10%	61g ± 3.00	54.67f ± 1.52	59.33g ± 1.52
20%	43d ± 1.00	47e ± 2.00	37.33c ± 2.51
30%	17a ± 2.00	21b ± 2.00	16.67a ± 1.52

Note: Values followed by different letters indicate significantly different results in the Duncan test (α=5%)

Table 3 shows that the average water content of instant butterfly pea flower powder ranges from 2.99% - 6.75%. The lowest average value of water content is found in the treatment of adding variations in maltodextrin content of 30% and a drying temperature of 90°C, which is 2.99%. While the highest average value of water content is in the treatment of adding variations in maltodextrin content of 10% and a drying temperature of 70° C, which is 6.75%. This is because maltodextrin generally has low hygroscopicity so that the hygroscopicity of microcapsules formed with increased maltodextrin concentration decreases, resulting in microcapsules with lower water content. At maltodextrin concentration of 10% to 20% with a drying temperature of 70°C, during the Duncan test there is no difference. This can happen if the air flow in the dryer at that time was too slow, causing the water vapor content around the dried material to became increasingly saturated as a result of the differences in vapor pressure inside and outside the ingredients (10% and 20% concentration) became small; this would have an impact on water evaporation, both treatments were no different. On the other hand, the water absorbed by maltodextrin evaporates more easily, so that the process of evaporating the powder water is easier and faster, causing the water content of the material to decrease (Arifin, 2006). The higher the drying temperature used, the higher the heat received by the powder so that the amount of evaporated water in the powder increases, and the measured water content becomes lower (Dwi, 2016). Differing collected results were reported by Ali et al. (2016), which showed that the water content of guava slices dried in a convection oven for 4.5 hours at a temperature of 80°C was 7.15%. Furthermore, the research conducted by Sarofa and Saraswati (2021) on watermelon extract enriched with butterfly pea flowers, with maltodextrin encapsulate at a temperature treatment of (40, 50, 60)°C and maltodextrin concentration of (10, 15, 20)%, drying time of 7 hours, resulted in water content of 3.15 - 4.91 %The research results showed that the water content value in instant butterfly pea flower powder was normal. This is still in accordance with the Indonesian National Standard (SNI) 01-3709-1995, which states that the maximum water content in spice powder is 12%. Meanwhile, the standard moisture content for spices according to USDA (2023) is between 4%-14%, depending on the type of spice.

Solution or Wetting Time

The average values of the dissolution time of instant butterfly pea flower powder with the addition of varying levels of maltodextrin and drying temperatures are presented in Table 4.

Table 4 shows that the average value of the longest dissolving time is found in the treatment of adding variations in maltodextrin content of 10% and a drying temperature of 70°C, which is 61 seconds. While the average value of the fastest dissolving time is found in the treatment of adding variations in maltodextrin content of 30% and drying temperature of 90°C, which is 16.67 seconds.

The interaction between variations in maltodextrin addition and drying temperatures can cause the dissolution time of instant butterfly pea flower powder to become faster. This is because maltodextrin is a carbohydrate that is classified as an oligosaccharide, which is easily soluble in water, so it can form a system that is evenly dispersed (Zhang et al., 2018). The greater the amount of maltodextrin added to the preparation, the faster the dissolving time. Cano-Chauca et al. (2005) reported that mango powder coated with maltodextrin had a solubility above 90%, indicating that maltodextrin can increase the solubility of the powder. This is due to its ability to be easily dispersed in a solution due to the presence of hydroxyl groups which tend to bind water to the granules. This is because polysaccharides, which are included as maltodextrin, have hydroxyl groups which are hydrophilic. The presence of hydroxyl groups also increases the solubility of organic compounds in water, because they can form hydrogen bonds with water molecules. According to Tako (2000), the presence of free hydroxyl groups will absorb water. Thus, the greater the number of hydroxyl groups in a polysaccharide molecule, the higher its ability to absorb water. According to Siregar (1992), in Husni et al. (2020), the time required to dissolve is around 1 (one) to 2 (two) minutes. Therefore, the quicker the dissolving time is, the better the quality of the instant butterfly pea flower powder will be.

Color Profile

Brightness Color Value (L)*

The average brightness (*L**) of instant butterfly pea flower powder treated with variations in maltodextrin content and drying temperature can be seen in Table 5.

Table 5 shows that the highest average level of brightness in instant butterfly pea flower powder treated with a maltodextrin content of 30% and a drying temperature of 90°C is 69.63 so that the quality of instant butterfly pea flower powder produced has a very strong level of brightness. The lowest level of brightness in instant butterfly pea flower powder with the addition of 10% maltodextrin content and a drying temperature of 70°C is 52, so that the quality of instant butterfly pea flower powder produced has a very low brightness level.

Based on the average values, the brightness level of instant butterfly pea flower powder tends to increase with increasing variations in maltodextrin content and drying temperatures. This is because maltodextrin has the characteristics of a white powder, so the brightness coordinate value *L** is influenced by the addition of

maltodextrin levels as an powder producing during the drying process. According to Wibawanto (2014), the higher the level of maltodextrin added, the higher the brightness coordinate (*L**) produced. So, the lower the maltodextrin content is added, the lower the brightness coordinate (*L**) obtained will be. Apart from that, maltodextrin is also able to form a layer around the color pigment, so the resulting powder will tend to be brighter. Meanwhile, for the drying temperature, the higher the temperature used, the more damage and loss of pigment in the material may occur (Oktaviana, 2012). So the brightness level value of instant butterfly pea flower powder increases and it becomes brighter. Another thing is that the *L** value is related to the pigment of the material, so the greater the pigment content is, the higher the absorption value and the lower the total reflectance will be, which can result in a lower *L** value. For the treatment of maltodextrin with the concentration of (20 and 30) % and drying temperature of (70 and 80) ˚C there was no statistical difference. This shows that at high maltodextrin concentrations of (20 and 30) %, it is still effective in preventing damage to the core material (anthocyanin pigment), especially up to the drying temperature of 80°C

In this study, the *L** results are related to the anthocyanin pigment content as shown in Table 2. That is, a high anthocyanin content produces powders with lower *L** values. Garcia-Estevez et al. (2017) reported that a low *L** value indicated a higher anthocyanin content, while an increase in the *L** value indicated a decrease in anthocyanin content.

Red/Green Color Value (a*)

The average red color (*a**) of instant butterfly pea flower powder treated with variations in maltodextrin content and drying temperature are presented in Table 6.

Table 6 shows that the average level of redness (*a**) is highest in instant butterfly pea flower powder treated with 10% maltodextrin content and drying temperatures of 70°C, which is at +2.6 so that the quality of the instant butterfly pea flower powder produced has a very strong level of redness. This is because more anthocyanin extract is added at a lower drying temperature. The higher the concentration of maltodextrin, the paler the product tends to be. As the color becomes less attractive, the panelists would find it more unlikeable (Sarofa and Saraswati, 2021). According to Lestari et al. (2019), maltodextrin has a white base color. When it is added to the extract it will increase the brightness of the product and so will reduce the *a** value and vice versa. On the other hand, in the anthocyanin concentration treatments of 20% and 30% with various drying treatments, the color tends to be green (negative sign). In this treatment, the maltodextrin content was 30% and the drying temperature was -3.23, so the quality of the instant butterfly pea flower powder produced has the lowest level of redness. The higher the concentration of maltodextrin, the impact will be greater on reducing the absorbance in the color test, because the wavelength changes or decreases so that the green color is absorbed more than the red color.

Based on the average values, the redness level of instant butterfly pea flower powder tends to increase and decrease with the high and low variations in maltodextrin content and drying temperature. This is because the value of the level of redness is used as an indicator of the color produced by a reddish or greenish sample. If the redness (*a**) is increasingly positive (+a), then it indicates that the powder produced is increasingly leaning towards red. If the reddish value is increasingly negative (-*a**), it indicates that the color of the powder is increasingly leaning towards green (Ernawati, 2010). Meanwhile, the level of redness is also influenced by the higher drying temperature treatment which can cause a decrease in the level of redness in instant butterfly pea flower powder. According to Nurhasanah (2015), in general, high drying temperatures can increase the loss of and damage to pigments in the material, and because anthocyanins are very sensitive to heat processes, the purple color of instant butterfly pea flower powder will fade due to degradation and polymerization. The treatment of adding variations in maltodextrin content and drying temperature had a real influence on the redness level of butterfly pea flower powder.

Yellow/Blue Color Value (b*)

The *b** notation of the chromatic color with mixed blue and yellow shows a +*b** (positive) value from 0 to +70 for yellow, and a –*b** (negative) value from 0 to -70 for blue.. The average sizes of the yellowish color (*b**) of instant butterfly pea flower powder treated with variations in maltodextrin content and drying temperatures are presented in Table 7.

Table 5. Average value of brightness color measurement (*L**) of instant butterfly pea flower powder

Maltodextrin Concentrations	Drying Temperatures		
	70°C	80°C	90°C
10%	52a ± 0.10	55.97b ± 0.45	57.47c ± 0.45
20%	58.4c ± 1.56	58.33c ± 0.35	64.2e ± 0.36
30%	62.77d ± 0.72	63.23d ± 1.10	69.63f ± 0.32

Note: Values followed by different letters indicate significantly different results in the Duncan test (α=5%)

Table 6. Average value of red color size (*a**) of instant butterfly pea flower powder

Maltodextrin Concentrations	Drying Temperatures		
	70°C	80°C	90°C
10%	+2.6e ± 0.45	+2.57e ± 0.30	+2.37e ± 0.10
20%	-2.27cd ± 0.37	-2.37cd ± 0.5	-2.47b ± 0.35
30%	-2.31cd ± 0.15	-2.42cb± 0.10	-3.23a ± 0.41

Note: Values followed by different letters indicate significantly different results in the Duncan test (α=5%); The (-) sign indicates the color direction tends to be green, the (+) sign indicates the color direction tends to be red

Table 7. Average value of yellowish color size (*b**) of instant butterfly pea flower powder

Maltodextrin Concentrations	Drying Temperatures		
	70°C	80°C	90°C
10%	$-11.16^{e} \pm 1.83$	$-11.17^{e} \pm 0.41$	$-12.33^{d} \pm 0.05$
20%	$-18.2^{c} \pm 0.20$	$-18.37^{c} \pm 0.35$	$-19.57^{ba} \pm 0.15$
30%	$-18.4^{b} \pm 0.70$	$-19.23^{ba} \pm 0.55$	$-21.27^{a} \pm 0.23$

Note: Values followed by different letters indicate significantly different results in the Duncan test (α=5%); The (-) sign indicates the color tends to be blue, the (+) sign indicates the color tends to be yellow

Table 7 shows that in, the yellowness value test (*b**) for all treatments shows a blue color, especially with the higher maltodextrin concentration and higher drying temperature. These results show that the color value *b** has a negative correlation with the results of the analysis of anthocyanin levels High temperatures cause the release of hydroxyl groups from maltodextrin so that the condition of the dried product becomes more alkaline. In alkaline conditions the anthocyanin pigment will turn blue, therefore when the drying temperature is increased the -b (blue) value also increases . This is in accordance with the research results of Shao et al. (2011) who reported that there was a negative correlation between the color variables *b** and *L** and the total anthocyanin content. Furthermore, it is stated that a negative correlation between the color variable *b** and total anthocyanins can occur if the value of the *b** variable is negative, that is, showing the color yellow to be moving to blue.

According to Harijono et al., (2001), adding maltodextrin levels can reduce the intensity of the yellow color so that the yellowness level value decreases due to the browning effect. The addition of more and more maltodextrin in the making of instant butterfly pea flower powder causes the yellowness level to become lower; this is because maltodextrin undergoes enzymatic and non-enzymatic browning reactions in the drying process. The color of the butterfly pea flower is purple, not blue, and the purple color is a combination of reddish coordinates (*a**) and yellowish coordinates (*b**), which are in the red and blue areas.

Conclusion

In chemical tests, treatment with a maltodextrin concentration of 10% and a drying temperature of 70°C obtained the highest antioxidant activity value of 51.47%, with the highest anthocyanin content of 47.36 mg/g.

In the physical test, treatment with a maltodextrin concentration of 30% and a drying temperature of 90°C produced the lowest water content of 2.99% (provided that all water content treatments still met SNI standards), the fastest dissolving time value was 16.67 seconds.

In the color profile test, treatment with a maltodextrin concentration of 10% and a drying temperature of 70°C obtained the lowest brightness measurement (*L**) of 52 ± 0.10, the highest redness measurement (*a**) of +2.6 ± 0.45, and the yellowish measurement (*b**) was relatively high at -11.16 ± 1.83 (note that for *b**all treatments point to blue).

The moisture content of all instant powder treatments when referring to instant spice powder standards, still complies with the Indonesian National Standards or the standards issued by the USDA.

The maltodextrin concentration of 10% at a drying temperature of 70ºC is the recommended treatment

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Investigation and Quantification of Phthalate Esters in Packaged Milk: A Study in Türkiye

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ABSTRACT

The aim of this study was to explore the concentrations of PAEs (Phthalate esters) in milk in Türkiye. For this purpose, a methodology was developed to quantify eight different PAEs in milk samples using a combination of dispersive solid-phase extraction (dSPE) and Liquid Chromatography coupled with Tandem Mass Spectrometry (LC-MS/MS). Employing this methodology, the concentrations of PAEs were evaluated in 34 milk samples. Results indicated the presence of PAEs in the milk samples; however, all tested compounds remained within the specific migration limits established by the EU. Among the analyzed PAEs, BBP (Benzyl butyl phthalate) was not detected in any samples, while DMP (di-methyl phthalate) (ND-5.51 µg/L) and DBP (di-butyl phthalate) (ND-7.91 µg/L) exhibited the lowest concentrations. DEHP (bis(2-ethylhexyl)) was identified as the most prevalent plasticizer with a maximum concentration of 41.31 µg/L. In conclusion, this study successfully investigated PAE concentrations in Turkish milk samples using a developed methodology. The results indicated the presence of PAEs within EU-established limits, with DEHP being the predominant plasticizer. Further research and monitoring efforts are crucial to ensure ongoing safety in packaged milk products.

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Introduction

Adequate and balanced nutrition is essential for the growth and development of the newborn and its protection from both communicable and non-communicable diseases in adult life (Chalupa-Krebzdak, Long, & Bohrer, 2018; Collard & McCormick, 2021). Milk and dairy products are among the most important food groups in a healthy and balanced diet (Pereira, 2014). Milk is a food that provides the physiological needs of mammals in the growing period and contains all the nutrients that play an important role in the nutrition of people of all ages (Silva & Smetana, 2022). These sensitive nutritional products are highly susceptible to chemical and microbial spoilage due to the rich nutritional elements and high water content they contain (Balthazar et al., 2022; Rejeesh & Anto, 2022). Therefore, it is packaged with packaging materials in order to facilitate the storage, transportation process and increase the shelf life (Cadwallader, Gerard, & Drake, 2023; Rejeesh & Anto, 2022). These packaging materials are polymer-based products and are produced by polymerization of many simple units called monomers, as well as additives such as heat and light stabilizers, colorants, lubricants, antioxidants (Arvanitoyannis & Kotsanopoulos, 2013). Additionally, plasticizers such as di-iso decyl phthalate (DIDP), di-n-octyl phthalate (DNOP), DEHP, di-isononyl-phthalate (DINP), DMP, DBP, di-ethyl phthalate (DEP), and BBP are employed to enhance the pliability and suppleness of packaging materials (Cirillo et al., 2015). Since phthalate esters do not have stable chemical bond interactions with the polymers they are included in, they can be separated from the polymer matrix under storage and some unsuitable conditions (vigorous shaking, high temperature, solar radiation etc.)(Yang et al., 2017) and human consumption of these foods may pose a human health concern for PAEs (Arfaeinia et al., 2020; Ebrahimi et al., 2016; Wang et al., 2015). The effects of PAEs on human health are regarded as endocrine disruptors, mainly because they interfere with the endocrine systems of living things (Pang, Skillen, Gunaratne, Rooney, & Robertson, 2021). Increasingly, epidemiological studies have shown that phthalate esters have hepatotoxic, teratogenic and carcinogenic properties, as well as many negative effects (Arbuckle et al., 2014). At the beginning of these effects are seen impairing spermatogenesis and decreasing sperm count in men (Matsumoto, Hirata-Koizumi, & Ema, 2008), allergic reactions with increased respiratory diseases in children (Buckley et al., 2018), prostate development in men, breast cancer formation in women, thyroid gland abnormalities (Arfaeinia et al., 2020). Based on the current literature, it has been reported that many foods contein PAEs such as in milk and dairy product (Dobaradaran et al., 2020; Mirzaei, Ahmadi, Shariatifar, & Ariaii, 2023), bottled water (Abtahi et al., 2019; Mehraie et al., 2022), carbonated soft drinks (Moazzen et al., 2018), infant formula (Isci, 2023) and juices

(Arfaeinia et al., 2020; Isci, 2024; Kargarghomsheh et al., 2023). Milk has the feature of being one of the basic foods for all age groups because it is cheap and easy to produce and contains the energy and nutrients needed by the body. Therefore, monitoring of commercial milk samples for potential contaminants such as PAEs is of great importance for consumer health and food safety (Dobaradaran et al., 2020). This study aims to analyze the packaged milk of all brands available in the Turkish market in terms of PAEs content and determine their compliance with legal regulations.

Materials and Methods

Chemicals And Reagents

The study utilized chemicals and standards of analytical grade. Formic acid (>98%, for analysis EMSURE® ACS, Reag. Ph Eur) and acetonitrile (>99.9%, OmniSolv® LC-MS) were supplied by Merck (Darmstadt, Germany). Di-isodecyl phthalate (DIDP), di-isononyl phthalate (DINP), and methanol (≥99.9%, hypergrade for LC-MS LiChrosolv®) were provided by Sigma-Aldrich (St. Louis, USA). The standard mixture, which included DEHP, di-n-octyl phthalate (DNOP), DMP, di-ethyl phthalate (DEP), DBP, and BBP esters, was sourced from Dr. Ehrenstorfer (Augsburg, Bavaria, Germany). Additionally, ISOLAB (Wertheim, Germany) supplied a 0.45 µm pore size PTFE filter, and RESTEK (Bellefonte, USA) provided the Q-sep ($MgSO_4$ (6000 mg), Sodium Acetate (1500 mg)) extraction salt and Q-sep dSPE tubes ($MgSO_4$ (1200 mg), PSA (400 mg), C18 (400 mg)) for extract cleanup. The selection of these materials was made with care to ensure the accuracy and reliability of the study results.

Sampling Method

A total of 34 milk samples were gathered from 10 international brands accessible in the Turkish market. The research encompassed all brands with products available for retail sale in Türkiye. Each sample was collected in triplicate and stored in a refrigerator until analysis. Milk samples were obtained from supermarkets and retail stores, representing commercially available milk products during the study period. The samples included different types of milk, such as unflavored whole milk (3.0% fat content), unflavored semi-skimmed milk (1.5% fat content), and flavored milk options like strawberry, cocoa, and banana. The analysis covered samples packaged in PET, cartons, and glass containers to assess potential variations in PAEs levels based on different packaging materials.

Sample Extraction Procedure

The determination of PAE contents in the samples was conducted using the methodology described by Isci et al. (2023), employing the dSPE technique. In adherence to this approach, ultrapure water and acetonitrile were introduced to the milk sample, and subsequently, dSPE extraction salt was added, followed by thorough vortexing. After centrifugation (4500 G; Thermo Fisher Scientific Inc.), the extracted supernatant (acetonitrile) was collected. After vacuum drying, cleaning salts were added to the tube, and the mixture underwent another round of centrifugation through vortexing. The resulting supernatant from the final step was then injected into the glass vial.

Instrument

The investigation utilized a triple quadrupole LC/MS system developed by Agilent Technologies based in Loveland, CO, USA. This system incorporated MS/MS capability and additional components, including a Vacuum Degasser, a quaternary pump, an Infinity Autosampler, and a thermostatted column oven. Chromatography procedures were executed using a 120 SB-C18 column (Poroshell, 3.0 mm, 100 mm, 2.7 µm), also procured from Agilent Technologies in Loveland, CO, USA.

LC-MS/MS Conditions and Analysis

The determination of PAEs involved both quantitative and qualitative assessments, employing the multiple reaction monitoring (MRM) mode with precursor-product ion transitions (Table 1 and Figure 1a,b). The LC-MS/MS was employed for this purpose, as depicted in Figure 1, which illustrates the LC-MS/MS MRM chromatogram of eight PAE's fragment ions with m/z ranging from 50 to 550 in milk. The LC-MS/MS system used a mobile phase A consisting of 0.1% formic acid + 5 mM ammonium formate in water, with an injection volume of 5 µl and a flow rate of 0.3 mL/min. Mobile phase B, prepared as 0.1% formic acid in methanol, was also applied. The total run time was 10 min. The LC-MS/MS instrument was configured with specific operational parameters, including a sheath gas flow rate of 10 L/min at a temperature of 400°C. The nebulizing gas flow pressure and temperature were constant at 50 psi and 300°C, respectively. The capillary ion spray voltage was set at 4.0kV, and the SB-C18 column was maintained at a consistent temperature of 40°C. To establish the calibration curve, various concentrations of PAEs (1, 5, 10, 25, 50, 75, 100, 250 µg/L) were injected twice into the LC-MS/MS system.

Table 1. Method verification parameters.

A	RT	SL	Linearly range		Recovery (%)		RSD (%)		Quantification		m/z	Reference ion
			(µg/L)	R^2	SM	WM	SM	WM	LOD (µg/L)	LOQ (µg/L)		
DMP	2.62	100	1.0-100	0.998	93.76	92.19	5.19	4.93	1.882	6.272	195.1>92.1	195.00>77.1
DEP	3.58	100	1.0-100	0.998	95.43	94.19	4.69	5.79	0.350	1.165	223.10>177.0	223.20>121.0
BBP	5.16	100	1.0-100	0.998	94.30	93.17	3.12	4.43	0.517	1.723	313.20>205.0	313.20>91.0
DBP	5.25	100	1.0-100	0.999	92.10	92.73	4.78	5.41	0.567	1.891	279.25>205.0	279.25>57.3
DEHP	7.92	100	1.0-100	0.999	97.24	93.45	6.19	5.73	1.176	3.920	391.30>279.2	391.10>167.0
DNOP	8.02	100	1.0-100	0.999	96.41	95.76	5.19	4.73	1.347	4.490	391.30>261.1	391.10>57.3
DINP	8.24	100	1.0-100	0.996	96.73	92.17	5.64	7.51	1.064	3.546	419.30>148.9	419.30>71.3
DIDP	8.51	100	1.0-100	0.999	95.31	96.15	4.70	5.42	0.474	1.581	447.40>148.9	447.40>85.2

A: Analytes; RT: Retention time (min); SL: Spiking level (µg/L); SM: Skim milk; WM: Whole milk

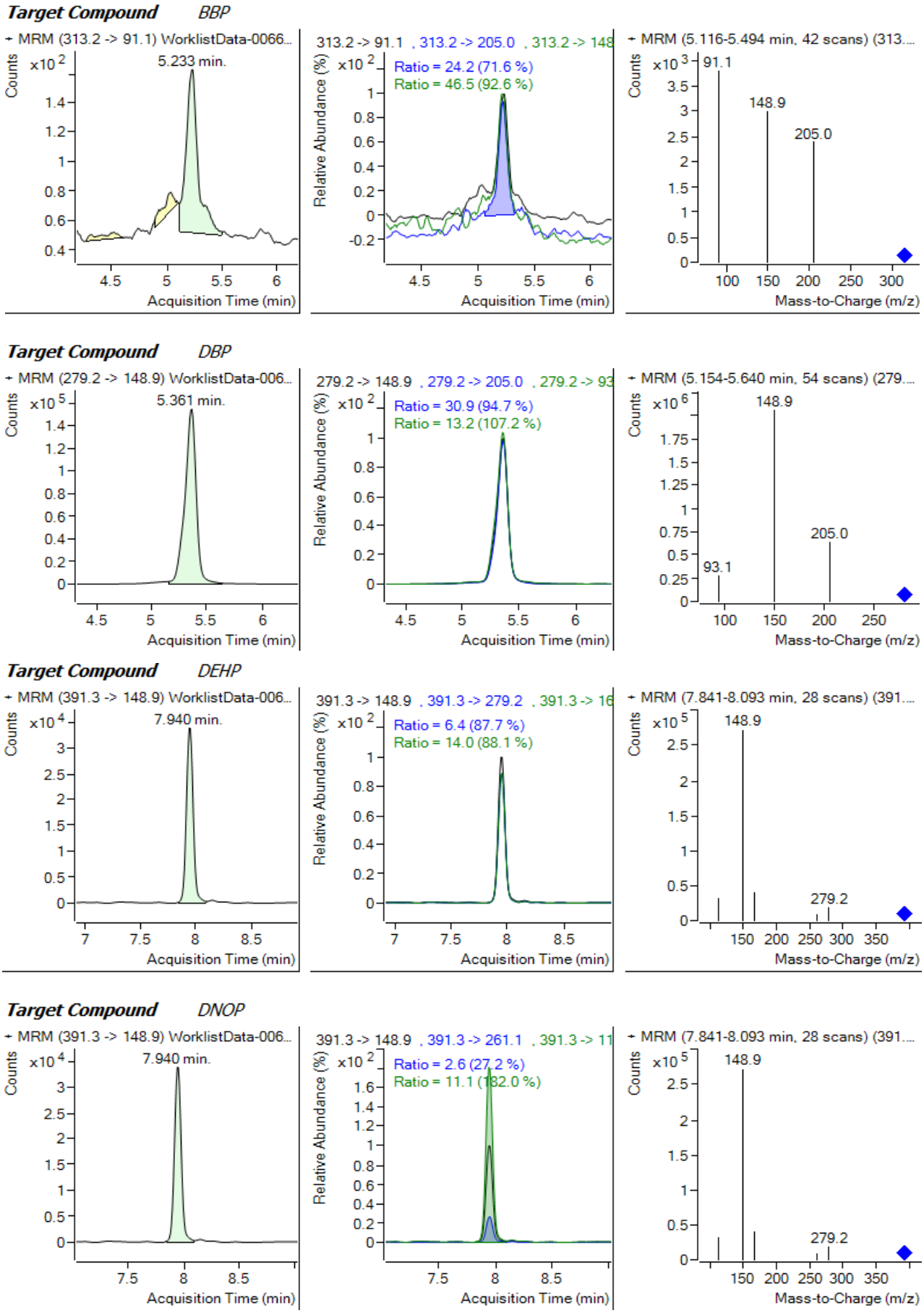

Figure 1a. LC-MS/MS MRM chromatogram of eight PAE's fragment ions m/z 50 and 550 in milk

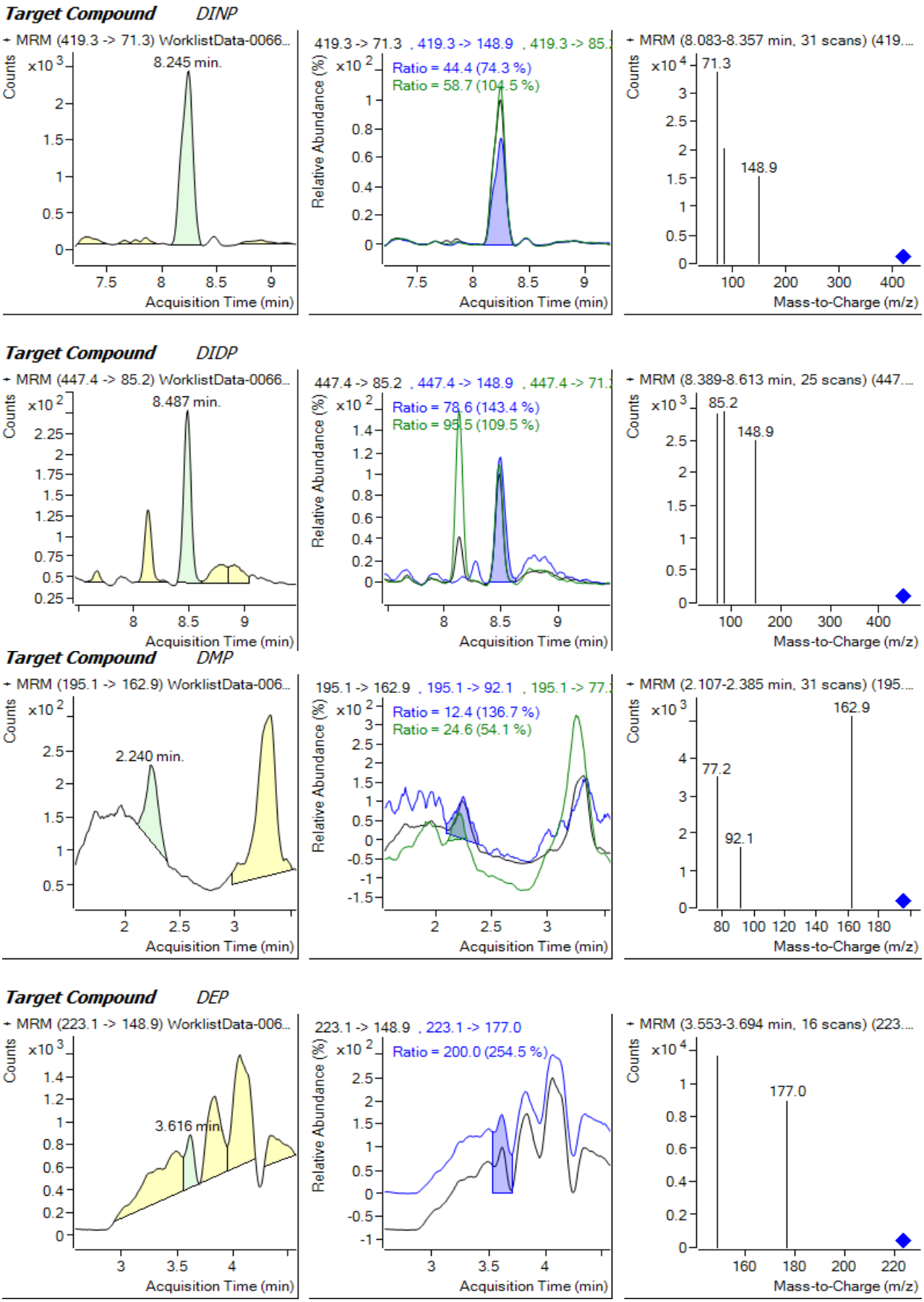

Figure 1b. LC-MS/MS MRM chromatogram of eight PAE's fragment ions m/z 50 and 550 in milk

Statistical Analysis

The statistical analysis of the results included applying PAEs and ANOVA (analysis of variance) to the samples, using SPSS version 26.0 (IBM, Chicago, IL, USA). Particularly for the milk samples, an analysis of variance was performed on the mean PAEs values, with a significance level set at $p < 0.05$. Duncan's multiple tests were employed to identify any statistical differences among the milk samples.

Results and discussion

Quality Control and Quality Assurance

The detected values of PAEs underwent correction by subtracting the average of blank values. To minimize the risk of contamination during PAEs analysis, precautions were taken to prevent contact between reagents, solutions, and plastic materials. Additionally, laboratory glassware was thoroughly cleaned with ultrapure water before utilization.

Table 1 demonstrates that the LOD and LOQ values for all PAEs were below 1.88 µg/L and 6.27 µg/L, respectively. Furthermore, the R^2 exceeded 0.995 for all PAEs. Furthermore, the recovery studies demonstrated results ranging from 92.1% to 97.2%, signifying a favorable recovery, with the RSD values for the target PAEs being under 7.51%. Comparing our results with relevant literature, specifically Kargarghomsheh et al. (2023), our LOD and LOQ values are lower, showcasing the sensitivity of our method. The recovery rates in our study align well with the recovery rates reported by Dobaradaran et al. (2020), affirming the consistency of our findings with established methodologies. In conclusion, our study not only demonstrates a meticulous laboratory protocol but also presents analytical results that are both sensitive and reliable.

PAEs Levels in Milk Samples

The PAE levels of 34 milk samples with different brands and packages which are available in Türkiye are shown in Table 2. All PAE compounds were detected in 100% of the milk samples except for BBP. The DEHP levels of whole milk (unflavored), strawberry flavored milk, cocoa flavored milk, banana flavored milk, semi-skimmed milk (unflavored) was detected to be within the range of <LOD-47.18 µg/L, <LOD-19.84 µg/L, 2.13-8.84 µg/L, 2.75-41.31 µg/L, and <LOD-33.12 µg/L, respectively. DEHP was determined as the most abundant plasticizer in milk samples and its amount in the samples also differed from each other. However, the highest average DEHP amount was found in unflavored milk (47.18 µg/L), while the lowest average value was found in semi-skimmed milk (1.21 µg/L). According to Commission Regulation (EU) No 10/(2011), the specific migration limit (SML) value of 1.5 mg/kg is established for DEHP (EU, 2011). When assessing the samples for compliance with the SML, it was found that the commercial milk samples did not exceed the SML for DEHP. Similar to the current study, DEHP levels in milk samples in plastic bottles were reported to be 198.84-622.37 ng/L by Dobaradaran et al. (2020), 41.3-228.6 ng/L by Mondal et al. (2022), ND-0.154 mg/kg by Kim et al. (2009).There are also some studies that detected higher DEHP levels, 25.0-247.0 µg/L by Selvaraj et al. (2016), 1.0- 936.0 µg/L by Jia et al.(2014), 187.0-201.0 µg/L by Farajzadeh et al. (2012), 13.14-242.39 µg/L by Feng et al.(2005). The PAE level of the same brand of flavored whole milks was statistically higher than the semi-skimmed unflavored milks ($P<0.05$). DEHP (7.73) with higher log partition coefficient (K_{ow}) is more likely to migrate into whole milk than other phthalates (DINP, DEP, DBP and BBP) with lower log K_{ow} (1.0-4.7) (Selvaraj et al., 2016). The DBP levels of whole milk (unflavored), strawberry flavored milk, cocoa flavored milk, banana flavored milk, semi-skimmed milk (unflavored) was detected to be within the range of ND-7.91 µg/L, ND-6.24 µg/L, ND-6.29 µg/L, ND-5.33 µg/L, and ND-4.37 µg/L, respectively. The highest mean DBP level was detected in unflavored milk (7.91µg/L), while the lowest mean concentration was detected in semi-skimmed milk (1.88 µg/L). Commission Regulation No 10/(2011), value of 0.3 mg/kg is reported for DBP (EU, 2011). When samples were evaluated for specific migration limits, commercial milk samples did not exceed the SML for DBP. When current studies are evaluated, DBP levels in plastic bottled milk samples were reported to be 105.0-498.0 ng/L by Dobaradaran et al. (2020), 3.30-150.2 ng/L by Mondal et al. (2022), 0.43-54.3 µg/L by Selvaraj et al. (2016), 99.0 µg/L by Kim et al. (2009), 4.0-10.0 µg/L by Feng et al. (2005). The levels of DINP in the different milk samples varied. For whole milk (unflavored), the detected levels ranged from ND to 28.82 µg/L. Similarly, strawberry flavored milk showed levels ranging from ND to 3.37 µg/L, while cocoa flavored milk exhibited levels between ND and 9.29 µg/L. Banana flavored milk had levels ranging from ND to 12.31 µg/L. Among the samples, semi-skimmed milk (unflavored) showed levels varying from ND to 12.42 µg/L. In the literature research, DINP has been studied in very few studies in packaged milk samples. The mean DINP level in this study is consistent with the mean level determined by Sørensen (2006) (5.0-12.0 µg/L). The DIDP levels of whole milk (unflavored), strawberry flavored milk, cocoa flavored milk, banana flavored milk, semi-skimmed milk (unflavored) was detected to be within the range of ND-8.43 µg/L, ND-9.84 µg/L, ND-10.77 µg/L, ND-2.22 µg/L, and ND-1.95 µg/L, respectively. The DIDP level of whole milk flavored are different from semi-skimmed milks. DIDP has not been investigated in milk and dairy products in the literature. However, according to Commission Regulation No 10/(2011), the SML for both DINP and DIDP is reported to be 9 mg/kg (EU, 2011). In this study, when the milk samples were evaluated for compliance with these SML values, it was found that the concentrations of DINP and DIDP in the commercial milk samples did not exceed the established limits. This suggests that the milk products examined in this study meet the regulatory requirements regarding the presence of DINP and DIDP. The BBP compound is very difficult to decompose under natural environmental conditions and is considered a carcinogen by the Integrated Risk Information System (IRIS) (1988), was not detected in any of the milk samples analyzed in this study. Similar to reported findings of Lin et al. (2015) reported that the BBP levels in three milk brands in plastic bottles ranged from ND-0.00 ng/L, <LOD (4.0 µg/L) by Sørensen (2006). There are also some studies that detected higher BBP level in milk samples

19.0- 85.0 µg/L by Jia et al. (2014), ND- 21.0 µg/L by Selvaraj et al. (2016), and ND-46.1 by Mondal et al. (2022). The DEP levels of whole milk (unflavored), strawberry flavored milk, cocoa flavored milk, banana flavored milk, semi-skimmed milk (unflavored) was detected to be within the range of ND-54.67 µg/L, ND-4.55 µg/L, ND-4.37 µg/L, ND-11.22 µg/L, and ND-9.51 µg/L, respectively. DEP concentrations of whole milk are different from each other, and DEP level of whole fat milks is higher than that of semi-skimmed milks. The highest DEP concentration was found in brand 1(54.67 µg/L) of the whole milk samples. When comparing our findings to previous studies, it is important to consider the range of results reported in the literature. Some studies, such as Farajzadeh et al. (2012), Lin et al. (2015), and Sajid et al. (2016), have reported similar values of (ND), indicating that the compound was below the limit of detection in their samples as well. On the other hand, studies conducted by Selvaraj et al. (2016) and Jia et al. (2014) have reported concentrations ranging from 0.43 µg/L to 54.3 µg/L and 13.0 µg/L, respectively, indicating the presence of the compound in those samples. Additionally, studies by Dobaradaran et al. (2020) and Mondal et al. (2022) have reported higher values ranging from ND to 16.24 ng/L and ND to 33.3 ng/L, respectively. These variations in results highlight the importance of considering different factors such as sample collection methods, analytical techniques, and geographical variations when comparing findings across studies. The DNOP levels of whole milk (unflavored), strawberry flavored milk, cocoa flavored milk, banana flavored milk, semi-skimmed milk (unflavored) was detected to be within the range of ND-5.56 µg/L, ND-4.17 µg/L, ND-5.31 µg/L, ND-4.13 µg/L, and ND-8.13 µg/L, respectively. The DNOP level of flavored whole milks is not different from semi-skimmed milk. The DNOP level was determined to be the highest in whole milk (8.13 µg/L). Most of the studies in the literature reported DNOP levels in commercial milk samples as below the detectable limit (Lin et al., 2015; Sajid et al., 2016). However, there are studies that found higher values ND-1795.00 ng/L (Dobaradaran et al., 2020). The DMP levels of whole milk (unflavored), strawberry flavored milk, cocoa flavored milk, banana flavored milk, semi-skimmed milk (unflavored) was detected to be within the range of <LOD, ND-4.18 µg/L, ND-5.51 µg/L, <LOD, and ND-2.22 µg/L, respectively. DMP level was determined to be the highest in cocoa flavored milk (5.51 µg/L). However, Herrero et al. (2021) reported as 0.43 µg/L. In another study Dobaradaran et al. (2020) reported as ND-16.24 ng/L. PAEs concentrations of different packaged milk samples examined in this study show differences. These differences may be caused by the flavoring and coloring agents used during production and the production method. In addition, it is seen that the machine milking and equipment used in the production processes of the products in the supply of raw materials and the packaging used in their sales can show great differences. The main sources of contamination of PAEs in packaged milk can be feeding of lactating animals with contaminated water and food, milking by machinery with plastic parts, containers used for the transport of milk and plastic packaging bottles (Fierens, Van Holderbeke, Willems, De Henauw, & Sioen, 2013).

Effect of Packaging Type on Paes Migration

The PAE concentrations determined in different packaging types are given in the Table 2. As can be seen, all level of PAEs expressed in µg/L of the 34 packaged milk samples analysed. Statistics analyses were conducted considering 34 milk samples in 10 brands and considering glass, PET, and cartons packaging type separately. The qualitative and quantitative levels of PAE found were different for PAEs and packaging (Figure 2). Total PAE concentration was determined in carton> PET> glass. The highest Total PAEs level were found for carton packaging (Brand1 in whole milk;153.53 µg/L). Carton food packaging includes on their inside PE, which protect the food content from the external factors, such as moisture, spoilage microorganisms (Bekhta, Lyutyy, Hiziroglu, & Ortynska, 2016). In this situation, these interior PE linings can release plasticizer, such as PAEs. In addition to food packaging material, different food additives (flavored, sweeteners, color additives), migration of PAEs from milk plastic transporting tank, automated milking machines (with PVC tubing) and processing apparatus (Fierens et al., 2012) may be the possible sources for higher PAEs observed in packed milk. Similar to the results of this research, Herrero et al. (2021) reported that highest total PAE level were found for metal pail (9094 pg/g f.w.) and carton packaging materials (8193 pg/g f.w.). The lowest Total PAE level were found for glass packaging. Glass materials of packaging does not contain polymer-based layers in their composition. Therefore, PAEs are not expected to be found in dairy products sold in glass packaging.

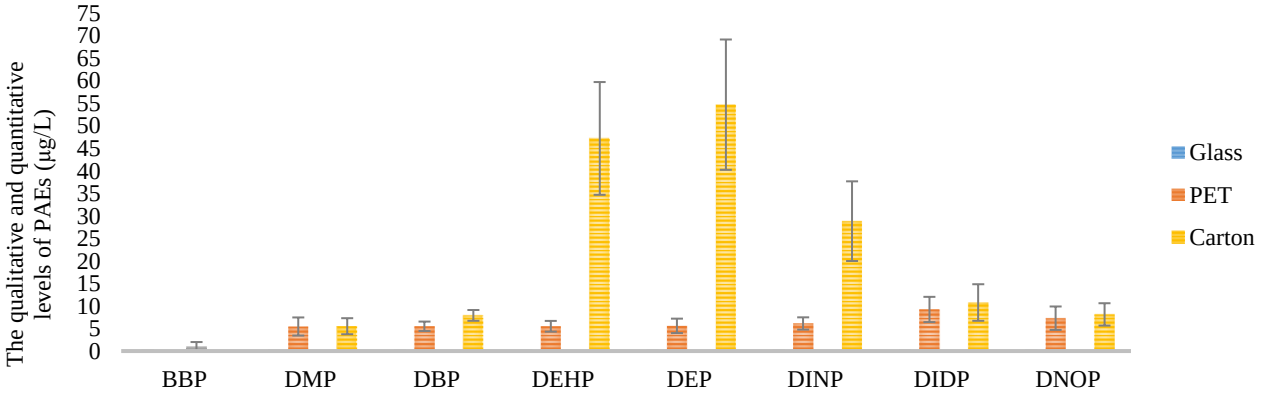

Figure 2. Concentration of PAEs in milk in different types of packing

Table 2. The PAE level of different milk samples (µg/L)

	Whole Milk Fat content (% 3.00)								
	BBP	DMP	DBP	DEHP	DEP	DINP	DIDP	DNOP	P
Unflavored									
Brand 1	ND	<LOD	7.91±1.16	47.18±9.11	54.67±33.27	28.82±2.21	8.35±8.28	5.34±5.97	C
Brand 2	ND	<LOD	4.95±1.31	8.30±3.78	2.75±1.56	4.63±1.33	6.86±7.74	4.68±5.23	C
Brand 3	ND	<LOD	4.83±2.35	2.54±1.20	2.45±1.68	4.42±1.65	8.43±8.83	5.56±6.17	C
Brand 4	ND	<LOD	4.35±2.56	2.37±1.12	2.72±1.15	3.16±1.30	7.76±8.18	5.40±5.93	PT
Brand 5	ND	<LOD	5.49±2.69	2.18±1.12	2.21±2.32	3.17±1.36	7.21±7.96	5.14±5.64	PT
Brand 6	ND	ND	ND	<LOD	ND	ND	ND	ND	G
Mean± SD	ND	<LOD	5.50±2.01	12.51±3.27	13.03±7.99	8.84±1.56	7.72±7.19	5.22±5.79	
(min. -max.)			(ND-7.91)	(<LOD-47.18)	(ND-54.67)	(ND-28.82)	(ND-8.35)	(ND-5.56)	
Strawberry flavored									
	BBP	DMP	DBP	DEHP	DEP	DINP	DIDP	DNOP	P
Brand 1	ND	2.51±2.79	3.36±2.38	2.13±1.45	2.62±2.52	2.19±1.14	8.85±10.52	2.63±0.28	C
Brand 2	ND	4.18±2.60	4.71±3.33	2.12±1.50	3.65±2.75	2.89±1.63	7.48±8.60	3.62±3.46	C
Brand 3	ND	3.10±2.96	3.93±2.78	5.55±2.56	4.55±2.44	3.37±1.97	9.84±12.60	3.85±2.98	C
Brand 4	ND	3.00±2.77	6.24±4.26	19.84±11.13	2.66±2.53	2.84±1.45	9.23±12.40	3.99±4.07	PT
Brand 5	ND	3.12±2.84	4.72±4.11	2.22±1.51	2.12±1.84	2.26±3.16	9.23±11.46	4.17±4.04	PT
Brand 6	ND	ND	ND	<LOD	ND	ND	ND	ND	G
Mean± SD	ND	3.18±2.79	4.59±3.37	6.39±3.63	3.12±2.42	2.71±1.58	8.93±11.12	3.65±2.97	
(min. -max.)		(ND-4.18)	(ND-6.24)	(<LOD-19.84)	(ND-4.55)	(ND-3.37)	(ND-9.84)	(ND-4.17)	
Cocoa flavored									
	BBP	DMP	DBP	DEHP	DEP	DINP	DIDP	DNOP	P
Brand 1	ND	5.51±9.63	4.77±1.78	8.34±3.94	2.53±1.59	3.25±1.71	8.60±9.48	ND	C
Brand 2	ND	4.12±9.09	3.25±1.52	2.70±1.13	3.17±2.13	2.53±1.4	9.69±8.99	3.55±8.71	C
Brand 3	ND	4.58±10.41	6.29±1.68	2.13±1.60	4.37±1.35	3.35±2.81	10.77±9.70	4.59±11.25	C
Brand 4	ND	3.93±9.26	5.83±1.80	7.93±2.90	3.91±1.36	9.29±2.83	8.79±10.15	5.12±12.53	PT
Brand 5	ND	5.45±8.73	4.14±2.93	2.60±1.33	3.90±2.17	6.12±2.84	8.66±10.24	5.31±13.02	PT
Brand 6	ND	ND	ND	2.54±1.70	ND	ND	ND	ND	G
Mean± SD	ND	4.73±9.60	4.86±1.94	4.74±2.17	3.52±1.72	4.91±2.32	9.72±9.71	4.64±11.36	
(min. -max.)		(ND-5.51)	(ND-6.29)	(2.13-8.84)	(ND-4.37)	(ND-9.29)	(ND-10.77)	(ND-5.31)	
Banana flavored									
	BBP	DMP	DBP	DEHP	DEP	DINP	DIDP	DNOP	P
Brand 1	ND	<LOD	3.16±2.23	2.75±1.13	6.51±2.37	8.65±4.70	1.93±1.72	4.13±1.28	C
Brand 2	ND	<LOD	3.16±1.96	24.13±6.24	6.60±2.43	11.19±4.39	1.98±1.85	3.87±1.94	C
Brand 3	ND	<LOD	3.47±2.45	41.31±28.17	11.22±5.48	12.31±7.29	2.22±2.02	2.86±1.09	C
Brand 4	ND	<LOD	5.33±3.20	15.17±8.37	6.20±2.61	5.30±1.76	2.00±1.79	2.81±0.74	PT
Brand 5	ND	<LOD	3.14±1.99	4.34±2.34	2.81±2.16	5.11±2.15	1.95±1.72	3.92±1.72	PT
Brand 6	ND	ND	ND	3.14±1.10	ND	ND	ND	ND	G
Mean± SD	ND	<LOD	3.65±2.39	17.52±9.26	6.66±3.00	8.51±4.06	2.01±1.82	3.52±1.35	
(min. -max.)			(ND-5.33)	(2.75-41.31)	(ND-11.22)	(ND-12.31)	(ND-2.22)	(ND-4.13)	
Semi-skimmed milk Fat content (% 1.50									
	BBP	DMP	DBP	DEHP	DEP	DINP	DIDP	DNOP	P
Brand 1	ND	<LOD	4.37±1.13	<LOD	2.20±1.54	1.76±1.88	1.79±1.63	8.13±0.06	C
Brand 2	ND	<LOD	1.88±1.56	1.93±1.67	1.53±2.95	2.45±1.96	1.81±1.62	2.80±0.09	C
Brand 3	ND	<LOD	2.73±1.85	1.21±1.93	2.26±2.33	1.29±1.13	1.74±1.53	2.51±0.04l	C
Brand 4	ND	<LOD	1.94±1.37	19.93±12.72	4.36±1.71	4.87±3.45	1.90±1.65	2.80±0.123	PT
Brand 5	ND	<LOD	2.13±1.41	1.90±1.17	1.97±2.22	1.13±1.80	1.95±1.47	2.45±0.07l	PT
Brand 6	ND	ND	ND	<LOD	ND	ND	ND	ND	G
Brand7	ND	<LOD	4.12±1.42	33.12±21.51	5.93±1.28	11.75±6.11	1.65±1.51	3.53±0.103	C
Brand8	ND	1.93±1.72	3.13±1.55	<LOD	6.33±2.27	3.59±1.87	1.48±1.31	3.50±0.05	PT
Brand9	ND	1.98±1.85	3.72±1.10	23.34±13.87	9.51±4.10	12.42±7.24	1.33±1.93	6.83±0.02	PT
Brand 10	ND	2.22±2.02	2.52±1.55	5.50±2.11	5.59±2.18	1.22±1.63	1.41±1.50	7.29±0.05	PT
Mean± SD	ND	2.04±4.24	2.93±1.53	9.60±6.31	4.38±2.18	4.34±2.93	1.74±1.60	4.36±0.03	
(min. -max.)		(ND-2.22)	(ND-4.37)	(<LOD-33.12)	(ND-9.51)	(ND-12.42)	(ND-2.37)	(ND-8.13)	

SD: Standart Deviation, ND, Not Detected; P: Packaging; C: Carton; PT; PET; G: Glass

However, It may be contaminated by lacquer on the cap, or from milking machinery and equipment used during production (Fierens et al., 2012). Average PAE levels of PET packaging materials lower than carton packaging materials and higher than glass packaging materials. The contributions of each PAEs to the total PAE level are shown in Table 2 and Figure 2. DEHP contributed the highest to the total PAE concentration, while DMP contributed the lowest. BBP, which has a carcinogenic effect, has not been detected in any packaging material. As a result, this study found that 34 commercial milk samples did not exceed the SML (specific migration limit) reported by the Commission Regulation (EU) No 10/2011. This monitoring is particularly important for plastic materials and articles intended for food contact to maintain consumer safety and prevent any potential risks associated with PAE exposure.

Conclusions

This research contributes novel insights into the occurrence of PAEs by examining milk samples in Türkiye. The results consistently demonstrate PAE levels in the milk samples that are below the standards established by the EU Regulation for all tested compounds, specifically adhering to the SML. DEHP and DEP emerged as the primary plasticizers among the analyzed PAEs. These findings underscore the significance of continuous monitoring and regulatory measures to safeguard the safety of packaged milk and address potential long-term exposure risks linked to PAEs. Persistent research and collaborative efforts are essential to reduce PAE concentrations in packaged milk, ultimately enhancing consumer safety in the long term.

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The Effect of Commercial Essential Oil Mixture Applied to Neonatal Simmental Calves on Growth, Development and Health Parameters

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ABSTRACT

This study, investigated the effectiveness of commercial essential oil mixture application in preventing calf losses due to diarrhea and on the growth, development and health parameters of calves. For this purpose, 24 newborn Simmental calves were used. Following birth, 20 ml of essential oil mixture was given orally via syringe to the calves in the treatment group after drinking milk in the morning for 5 days. The average birth weight of calves was 39.0 ± 0.72 kg. Birth weights of male and female calves were 42.3 ± 1.01 and 38.6 ± 0.96 kg respectively, the difference between groups were found to be significant. First month weight was 45.7 ± 1.67 and 42.4 ± 1.65 kg in the control and treatment groups, respectively. There was no difference between the control and treatment groups in terms of body measurements taken at birth, but a significant difference was observed in chest circumference in favor of the control group in terms of measurements obtained at the 1st month. There was no difference between the treatment and control groups in terms of hematological parameters detected in the blood taken on the 10th day and in the 1-month period. No differences were observed between groups in terms of immunoglobulin levels (IgM and IgG). The commercial essential oil mixture had no effect on the fecal score of the calves included in the trial. It was revealed that the essential oil mixture does not make any difference in the growth, development, and health of Simmental calves in a one-month period. In future studies on the subject, it is recommended that higher doses of the essential oil mixture be investigated.

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Introduction

In dairy cattle enterprises, 60% of the total income consists of milk, and 40% is obtained from the calf sales (Demir et al., 2019). However, in some regions, especially Eastern Anatolia, due to the inadequacy of the cold chain infrastructure in the preservation of raw milk and the problems experienced in marketing, calf sales are the main source of income for the enterprises. There has been an increase in calf deaths both in the world and in Turkey in recent years (Bayram et al., 2016a; Bleul, 2011). While the mortality rate in dairy calves is reported to be 10% worldwide, it was reported to be over 15% in Turkey (Şahal et al., 2018).

It is estimated that approximately 900 thousand to 1 million calves die every year in Turkey (Şahal et al., 2018; Günlü, 2018). In a recent study (Günlü, 2018), it was reported that 5 594 000 calves were born in Turkey in 2018, and approximately 17.6% of these calves (987 000 heads) died. In order to meet the need for breeding heifers and beef cattle in dairy and beef cattle enterprises as a result of high calf losses, huge expenditures have been made/are being made to import live animals. According to official reports, in 2018, 1 460 741 heads of cattle were imported, 116 081 of these cattle were used for breeding, 1 211 756 heads were used for fattening and 132 904 heads were slaughtered and used in the meat industry, and the total cost of this import was reported to be 1 692 068 152 US dollars to Turkey (Günlü, 2018). The current average prices of one-week-old Holstein-Friesian, Brown-Swiss and Simmental calves were reported to be 13, 19 and 23 thousand TL, respectively (Anonymous, 2023). Both, high average calf prices and animal imports as a result of high calf losses leading to serious economic burdens for the cattle enterprises in Turkey. At least 25% of these losses could be prevented with effective care, nutrition and protection measures for the calf and the dams in cattle farms. According to current data, average carcass obtained from cattle in Turkey is 296 kg/head (Anonymous, 2022), reducing calf losses by 25% will considerably increase the red meat production and thus, animal based protein consumed per person.

The majority of calf deaths (80%) occur in the birth-28-day period, this period is called the neonatal period (Karslı & Evci, 2018). The most significant reasons for calf losses in this period are calf diarrhea (45.2%), respiratory tract (22.2%), and foot and mouth disease (19.3%) (Demir et al., 2019). The causes of calf diarrhea are generally classified as microbial (infectious) and non-microbial (non-infectious). While infectious diarrhea is caused by parasitic, viral or microbial pathogens, non-infectious diarrhea occurs as a result of inadequate environmental conditions and nutrition (Karslı & Evci, 2018). The most fundamental reason for calf losses due to diarrhea is that newborn calves are not given timely and sufficient amount of colostrum. A study conducted in the USA revealed that the main reason for approximately one third (31%) of the calf losses within a week after birth was the insufficient passive immunity, which is caused by not receiving enough colostrum (Walsh et al., 2007). For newborn calves to have sufficient passive immunity serum Ig levels must be above 10 µg/ml (Vicente et al., 2014).

In the cattle industry, antibiotics have been widely used to prevent diarrhea and accelerate growth and development in calves. However, as a result of scientific evidences presenting that frequent use of antibiotics results in antibiotic resistance in disease-causing microorganisms and creates residues in animal-based products, thus its use as a growth factor in animal nutrition was first banned in Sweden in 1986, in all EU member countries in 2005, and in Turkey on January 1, 2006 (Ünlü & Erkek, 2013). These bans, both in Turkey and other countries, have triggered scientists to search for new safe and natural feed additives as an alternative to antibiotics (Özdemir et al., 2022). Essential oils stand out among alternative products with their wide range of proven benefits to health. As a natural product essential oils are reported to be effective in preventing diarrhea, modifying intestinal microflora as well as increasing feed utilization and daily live weight gain in calves (Uetaka, 2013; Jounany & Morgavi, 2007).

There are two different opinions regarding the effectiveness of essential oils on calves. First is increasing the utilization of nutrients as a result of increased enzyme amount and activity due to the stimulation of endogenous enzymes, and the second is protecting animal health by regulating the gut microbial flora (Bilgin & Kocabağlı, 2010; Zhang et al., 2005). Thymol, carvacrol, and eugenol are reported to be the major components of essential oils as a result of chemical analysis. The composition of essential oils varies depending on the geographical feature of the plant from which it is obtained. Especially carvacrol the component found in essential oils has a strong antimicrobial effect even at low concentrations (Bilgin & Kocabağlı, 2010; Dusan et al., 2006). Essential oils are known for their antiseptic, antioxidant, digestive stimulant, antimicrobial and enzymatic effects. Due to these properties, various studies have been carried out to investigate the effects of essential oils in protecting animal health and increasing the development and performance. In some of these studies, essential oils are reported to control harmful and disease-causing bacteria in the rumen and intestines and inactivate them (Selvi, 2018; Sağdıç & Özcan, 2013).

This study investigated, the effects of a commercial essential oil mixture obtained from 27 different aromatic medicinal plants, mainly thyme, clove, eucalyptus oil and licorice, on growth, development and health parameters in neonatal Simmental calves.

Material and Method

Animal Material

The animal material of the study consisted of Simmental calves born in Atatürk University Food and Livestock Research and Application Center Cattle Breeding Unit. Calves were divided into two groups: control (12 heads) and treatment (12 heads). Following the routine practices specified in the enterprise and colostrum feeding the calves were taken to the individual pens. The calves in the treatment group were fed with 20 ml of commercial essential oil mixture orally via syringe, immediately after the morning milk feeding, for 5 days, starting from the 3rd day of birth. All the experimental protocols adhered to and were approved by the guidelines of the Animal Ethics Committee of Ataturk University (Approval date: 11 August 2022; Decision No.: 2022/154).

Feeding

The calves, were taken into individual pens, and fed with whole milk obtained from the Atatürk University Food and Livestock Research and Application Center Cattle Breeding Unit. The milk given to the calves kept constant at 10% of their birth weight. Calf starter feed was offered to the calves starting from one week of age. The starter was purchased from a commercial feed mill. In addition to the starter dry hay was given to the calves. Dry hay was obtained from Atatürk University Plant Production Application and Research Center. The nutritional contents of the liquid and solid feeds used in the study are given in Table 1.

Table 1. Composition of solid and liquid feeds used in the study

Nutrients (%)	Milk	Calf Starter	Dry Hay
Dry matter	12.0	89.8	93.1
Crude Protein	3.8	19.1	7.1
Ether Extract	4.1	4.8	3.8
Crude Ash	0.7	8.0	8.4
Crude Cellulose	_	12.0	28.1

The commercial essential oil mixture was purchased from a private company, the essential oil mixture contained oils from 27 different aromatic medicinal plants (TERASİNN-TR33-K-026869/09.04.2018). TERASINN, which is a completely herbal product, contains pectin, essential oil mixture and betane. Thyme, clove, eucalyptus oil and licorice root, were the main components found in the product. The component of the product consists of carvacrol, 1.8 cineole as well as organic minerals. 20 ml of commercial essential oil mixture was given to newborn calves orally following the morning milk feeding for 5 days by using a syringe and serum hose.

Method

The calves included in the trial were examined in terms of various parameters. These parameters are given below:

Live weight and various body measurements (height at withers, chest depth, heart girth, body length, cannon bone girth) were determined at birth and one month of age. In order to determine the effect of commercial essential oil mixture on diarrhea, fecal consistency scoring method developed by Larson et al. (1977) was utilized. In accordance with the mentioned method, the classification

of the manure of the calves were performed as follows; 1: normal, soft, solid consistency, not fluid, 2: soft, semi-solid, mostly solid, 3: fluid, semi-solid, mostly fluid, 4: aqueous, completely fluid.

Blood and Serum Sampling

Blood samples were collected from the serum tubes without anticoagulant (8 ml) and K2 EDTA tubes (3 ml) from the jugular vein of the calves. Hematological analysis were performed on blood samples taken into EDTA tubes. Blood samples were centrifuged at 3500 rpm for 10 minutes to separate plasma and serum. Subsequently, serum was transferred to eppendorf tubes and calf numbers and sample collection dates were written on them. Immunoglobulin analyses were made on serum samples separated from the whole blood. Blood serums were kept at -20 °C for 24 hours, then frozen and stored in a deep freezer at -80 °C until biochemical analysis was performed.

Hematological and Biochemical Analyzes

Blood samples taken from the calves first within 24 hours following birth, then on the 10th day and at the 1-month of age was utilized for hematological analysis. The blood samples taken were counted on the same day using the Abacus Junior Vet5 hemogram device in the Laboratory of Internal Medicine Department of Atatürk University Faculty of Veterinary Medicine. White blood cell (WBC), lymphocyte (LYM%), neutrophil (NEU%), red blood cells (RBC) and hematocrit (HCT) values were determined and recorded. Serum IgM and IgG analysis were performed in accordance with the procedure described by the manufacturer (Bioassay Technology Laboratory).

Statistical Analysis

GLM procedure available in the SPSS 20.0 (SPSS, 2013) package program was utilized to analyze the data obtained. In the research, analysis of variance was performed using a 2×2 factorial experimental plan. The data conformed to normal distribution. In the statistical model, commercial essential oil mixture and calf gender were included in the model as factors. To eliminate the effects of colostrum quality resulting from birth order, calves born from heifers were used in the study. Non-parametric fecal consistency scores, which are scored categorically as 1, 2, 3, and 4 were analyzed according to the Chi-Square (X2) independence test (Yıldız & Bircan, 1994).

Results and Discussion

Weights and Body Measurements

Birth weight is a significant factor affecting growth and development of calves in the postnatal period and is also used as a selection criterion in cattle farms. Birth and 1st month weights of Simmental calves are given in Table 2.

Simmental calves' average birth weight was 39.9 ± 0.72 kg. While average birth weight of the calves was close to the values reported by Koçak et al. (2008), it was lower than the results of similar studies (41.5-42.1 kg) (Özen, 2022; Aydoğdu & Karslı, 2020; Baykan, 2016). To eliminate the effect of maternal age, only the calves obtained from heifers included in the study. This might be the primary reason for the lower calf birth weights observed in this study.

The average birth weight of the control and treatment groups were determined as 39.9 ± 0.74 and 39.3 ± 1.30 kg, respectively. There was no statistically significant difference in terms of the birth weights between groups.

The male and female calves' birth weights were 42.3 ± 1.01 and 38.6 ± 0.96 kg, respectively. Male calves were determined to be 3.7 kg heavier than the female calves at birth ($P<0.05$). The findings of the study are comparable with the results of various studies conducted on Simmental calves (Baykan, 2016; Koçak et al., 2008; Özlütürk et al., 2006; Kaygısız, 1998).

The study's average 1st-month weight of Simmental calves was 44.0 ± 1.00 kg. One-month weight of the calves determined in this study was found to be lower than the average 1st month weights reported for Simmental calves (49.5-62.0 kg). The first reason for lower one-month weight of Simmental calves obtained in this study may be slower growth performance of the calves having lower birth weight. And the second reason for this result is thought to be the effect of the season since the study was carried out under harsh winter conditions of Erzurum Province, which is located over 2000 m from the sea level.

Average 1st month weights in the control and treatment groups were determined as 45.7 ± 1.19 and 42.4 ± 1.65 kg, respectively. Control group calves had 3.3 kg higher weight at one month of age compared to the calves in the treatment group, however, this difference was determined to be statistically insignificant. In other words, the commercial essential oil mixture used in the study had no significant effect on the growth performance of calves. Similarly, previous studies investigating the effects of thyme oil (Selvi, 2018; Ünlü & Erkek, 2013), laurel oil (İzzadden & Kaygısız, 2018), and thyme juice (Özkaya et al., 2018) on the growth and development of calves showed no significant difference in the weights of calves in the first month or weaning period. In contrast, Liu et al. (2020) reported that the addition of essential oil to the ration resulted in a significant increase in the 1st month weight of Holstein calves. They noted that essential oils extracted from plants had a positive effect on growth performance by increasing feed consumption.

The average 1st month weight was determined as 46.6 ± 1.67 kg in male and 43.2 ± 1.10 kg in female calves, and the difference of 3.4 kg in favor of male calves was statistically insignificant. The findings of the study obtained are compatible with the results of Selvi (2018) who reported 44.3 kg and 42.6 kg first month weight for male and female calves, respectively. Furthermore, İzzadden and Kaygısız (2018) also noted that the effect of gender on 1st month weight of calves was statistically insignificant. They reported that one-month weight of the calves were 58.2 kg and 54.2 kg for male and female calves, respectively.

Body Measurements at Birth and 1st Month of Age

Increases in body measurements of calves are critical parameters in determining the growth and skeletal development of calves. Some body measurements of Simmental calves determined at birth and in the first month are given in Table 3. There was no significant difference between the control and treatment groups in terms of body measurements determined at birth. Nevertheless, the height at withers cannon bone girth of male calves were determined to be 2.1 and 1.1 cm higher than female calves, respectively. The differences between sex groups were statistically significant ($P<0.05$).

Table 2. Birth and 1st-month weights of the calves in control and treatment groups

	Group			Sex		
	Control (N=12) X ± Sx	Treatment (N=12) X ± Sx	S	Female (N=12) X ± Sx	Male (N=12) X ± Sx	S
Birth Weight	39.9 ± 0.74	39.3 ± 1.30	NS	38.6 ± 0.96[b]	42.3 ± 1.01[a]	*
1st month Weight	45.7 ± 1.67	42.4 ±1.65	NS	43.2 ± 1.10	46.6 ± 2.67	NS

S = Significance NS = Not Significant *P<0.05

Table 3. Body measurements at birth and 1st month of age

Body measurements (cm)	Control (N=12) X ± Sx	Treatment (N=12) X ± Sx	S	Female (N = 12) X ± Sx	Male (N=12) X ± Sx	S
Birth						
Height at withers	68.3 ± 0.63	68.7 ± 0.79	NS	67.4 ± 0.56[b]	69.5 ± 0.71[a]	**
Chest Depth	33.0 ± 0.52	32.7 ± 0.27	NS	32.5 ± 0.49	33.2 ± 0.29	NS
Chest Girth	74.5 ± 0.53	75.2 ± 0.98	NS	73.8 ± 0.82	75.8 ± 0.70	NS
Cannon bone girth	14.9 ± 0.28	15.0 ± 0.21	NS	14.4 ± 0.21[b]	15.5 ± 0,16[a]	**
Body Length	68.4 ± 0.96	66.7 ± 0.89	NS	66.6 ± 0.96	68.3 ± 0.89	NS
1 month of age						
Height at withers	72.8 ± 0.75	71.4 ± 0.56	NS	71.2 ± 0.47	73.5 ± 0.85	NS
Chest Depth	35.3 ± 0.36	34.5 ± 0.63	NS	34.7 ± 0.40	35.3 ± 0.54	NS
Chest Girth	80.5 ± 1.28[a]	75.4 ± 1.50[b]	*	76.5 ± 1.04[b]	81.1 ± 1.79[a]	*
Cannon bone girth	15.2 ± 0.29	14.2 ± 0.38	NS	14.1 ± 0.16[b]	15.7 ± 0.28[a]	**
Body Length	74.9 ± 1.29	71.9 ± 1.86	NS	72.2 ± 1.53	75.6 ± 1.37	NS

* = P < 0.05; **= P< 0.01; S = Significance, NS=Not Significant

Table 4. Hematological parameters at birth, 10-day and 1-month-of ages of calves

Hematological Parameters	Control (N=12) X ± Sx	Treatment (N=12) X ± Sx	S	Reference Range
Birth				
WBC (White Blood Cell)	8.79 ±1.13	7.95 ±1.01	NS	4 - 12
LYM (%) (Lymphocyte)	3.46 ±0.38	3.80 ±0.46	NS	2.5 - 7.5
NEU (%) (Neutrophil)	4.71 ±0.70	3.54 ±0.54	NS	0.6 - 6.7
RBC (Red Blood Cell)	9.60 ±0.30	8.25 ±0.35	**	5 - 10
HCT (Hematocrit)	36.05 ±1.31	32.58 ±1.55	NS	24 - 46
10 days of age				
WBC (White Blood Cell)	11.39 ±1.44	12.34 ±0.88	NS	4 - 12
LYM (%) (Lymphocyte)	5.02 ±0.59	5.35 ±0.55	NS	2.5 - 7.5
NEU (%) (Neutrophil)	5.65 ±0.94	6.17 ±0.57	NS	0.6 - 6.7
RBC (Red Blood Cell)	9.36 ±0.36	8.98 ±0.44	NS	5 - 10
HCT (Hematocrit)	32.42 ±1.53	33.54 ±1.76	NS	24 - 46
1 month of age				
WBC (White Blood Cell)	11.19 ±1.48	12.43 ±0.92	NS	4 - 12
LYM (%) (Lymphocyte)	5.08 ±0.34	5.54 ±0.21	NS	2.5 - 7.5
NEU (%) (Neutrophil)	5.62 ±1.22	6.29 ±0.84	NS	0.6 - 6.7
RBC (Red Blood Cell)	9.40 ±0.35	9.87 ±0.71	NS	5 - 10
HCT (Hematocrit)	28.85 ±1.22	28.86 ±2.09	NS	24 - 46

S = Significance NS = Not Significant * = P < 0.05 **= P < 0.01

The differences in control and treatment groups for body measurements at one month of age were determined to be statistically insignificant except for the chest girth. Chest girth of the calves in the control group was 5.1 cm higher than the treatment group (P<0.01). In addition, chest girth (P<0.05) and cannon bone girth (P<0.01) of the male calves were significantly higher than female calves at one month of age.

Hematological Parameters

Having hematological values in the reference range is critical for calves to be healthy and show ideal growth and development performance (Zwald et al., 2004). Detection of the number and ratio of certain cells in the blood plays a significant role in the early diagnosis of infections in calves.

The results of hematological analysis performed on blood samples taken from Simmental calves at birth, 10th day and 1 month of age are given in Table 4. The analysis results showed that the hematological values of both control and treatment groups were within the reported reference range in the blood samples taken following the birth. Even though the RBC (Red Blood Cell) value in the blood obtained right after birth is within the reference range, control group calves had a significantly higher RBC count than the calves in the treatment group (P<0.01). The differences observed for other hematological values (WBC, LYM, NEU, HCT) were determined to be insignificant.

There was no significant difference between the control and treatment groups in terms of the hematological values

obtained from the blood samples taken at the 10-day and 1-month of ages of calves. In other words, the commercial essential oil mixture did not have a significant effect on blood parameters of Simmental calves. All parameters determined in the control and treatment groups during the ten-day and one-month period were within the reference ranges.

A similar study conducted on calves with and without diarrhea (Çorapsız, 2023), showed that diarrhea did not cause a significant difference in body temperature and live weight of calves, and there was no significant difference between the two groups in terms of white blood cells and lymphocytes counts. In another study conducted to determine the effect of different etiological factors on hemogram parameters in calves with diarrhea during the neonatal period, Atçalı and Yıldız (2020) reported that the WBC (leukocyte) of calves with diarrhea was significantly higher ($P<0.05$) than the control group calves and no significant difference was determined between two groups in terms of LYM, RBC and HCT levels. Comparable results for blood parameters were also reported in studies aimed to determine the effects of plant essential oil supplementation to ration (Özkaya et al., 2018; Ünlü & Erkek, 2013).

Immunoglobulin M and G Levels

Immunoglobulins (Ig) are glycoproteins produced by plasma cells and have crucial functions against diseases. Ig (IgG, IgM) levels detected in the blood serum of Simmental calves at birth, 10th day, and 1 month of age are presented in Table 5. IgM are the first antibodies that respond to body pathogens. As a result of the biochemical analysis, no significant difference was found between IgM levels of calves at birth, 10 days and 1 month of ages. Although the IgM mean of the treatment group was higher, this difference was statistically insignificant. In this study 10th day IgM level determined for both the control (0.6069) and treatment groups (0.9147) was considerably lower than the results reported in two similar studies (3.17-4.30) (Bayram et al., 2016b; Akbulut et al., 2003).

IgG is the most abundant antibody in the body, but has the smallest molecule. First, IgG binds to important pathogen surface proteins and inactivates pathogens such as viruses and bacteria, preventing the pathogen from interacting with host cells. Consequently, the antibody neutralizes the pathogen's ability to enter and replicate in its host cells (Thompson, 2016). No statistically significant difference was determined between the IgG levels of Simmental calves at birth, 10th day and 1 month of age. Although the IgG levels were higher in calves in the treatment group, the difference between groups was insignificant. In this study, IgG levels detected on both the 10th day and the 1st month were considerably lower than the average values reported in previous studies on the subject (18.4-21.26) (Baykan, 2016; Akbulut et al., 2003). Comparably, Liu et al. (2020) reported that IgG and IgM levels increased significantly ($P<0.01$) with essential oils supplementation to ration in calves.

Mazengera et al. (1985) and Gilbert et al. (1988) reported that mean serum IgG concentrations were 24.0 mg/ml for the Simmental calf at 36 hours of age. Compared to the relevant result, the result obtained in this study considerably lower (4.34 mg/L and 4.59 mg/L). Furthermore, Liu et al. (2020) reported that calves fed with essential oil combination at 44.1 ppm had higher IgG and IgM levels on days 14, 28, and 42 than the control group.

Fecal Consistency Score

Calf diarrhea is among the most prominent causes of early calf diseases and losses (Urie et al., 2018). Calf diarrhea also results in a decrease in growth performance and a delay in the age of first calving in cattle's early life (Windeyer et al., 2014). In the current study fecal consistency scoring method developed by Larson et al. (1977) were used to determine the diarrheal condition of Simmental calves. Fecal scores of the control and treatment group calves were scored on a scale of 1 to 4, and the results are given in Table 6.

According to the result of the fecal consistency scoring, the difference between the treatment and control group was found to be insignificant ($P > 0.05$). The commercial essential oil mixture seems ineffective on the calves fecal consistency scores. In contrast, results of the previous studies (İzzadden & Kaygısız, 2018; Selvi, 2018; Ghosh et al., 2010), indicated to significant effect of essential oils on the fecal scores through a positive effect on the intestinal microbial population (Ghosh et al., 2010). Also, Katsoulos et al. (2022) reported that use of Greek thyme essential oil mixture significantly reduced the fecal score.

Table 5. Birth, 10th day and 1 month IgM and IgG levels (µg/ml) of calves

Period	IgM		S	IgG		S
	Control	Treatment		Kontrol	Muamele	
Birth	0.4703±0.07553	0.4712±0.0793	NS	4.3455±0.8155	4.5901±0.7912	NS
10. day	0.6069±0.0719	0.9147±0.01921	NS	6.1690±1.3890	7.9971±1.3479	NS
1. month	0.9541±0.1272	0.9760±0.1134	NS	8.0156±0.3278	10.3278±2.5038	NS

Ig: Immunoglobulin S: Significance NS: Not Significant

Table 6. Fecal consistency scores of the control and treatment group calves

			Fecal Score				Total
			1	2	3	4	
Group	Control	Frequency (N= 112)	65	25	19	3	112
		Percentage in the group %	58.0%	22.3%	17.0%	2.7%	100.0%
	Treatment	Frequency (N= 59)	24	17	15	3	59
		Percentage in the group %	40.7%	28.8%	25.4%	5.1%	100.0%
Total		Frequency (N= 171)	89	42	34	6	171
		Percentage in the group %	52.0%	24.6%	19.9%	3.5%	100.0%

Conclusion

According to the results obtained from the study, the commercial essential oil mixture, which was investigated to be a potential alternative to antibiotics in the prevention of neonatal calf diarrhoea, which causes a significant decrease in calf growth, development as well as health parameters and results in a considerable economic loss in the enterprises, showed no significant effect. Furthermore, there was no significant difference on the growth, development, and health of Simmental breed calves. Further, long-term studies investigating different doses of this product should be conducted to clearly understand the effects of the used essential oil mixture.

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This study is summarised from Fatma EMİR's master's thesis

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Management of Root-Knot Nematode (*Meloidogyne* spp.) on Kiwifruit Seedlings using Different Plant Extracts, Biocontrol Agents, and Chemical Nematicides

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ABSTRACT

Root-knot nematodes (RKN), (*Meloidogyne* spp.), are the major biotic factor responsible for the limiting production of Kiwifruit in Nepal including Kiwifruit orchard of Warm Temperate Horticulture Center, Nepal. Hence, there is a pressing demand for nematicides that are both easily accessible and cost-effective while being environmentally friendly. A screenhouse experiment was conducted in the Summer of 2023 with an objective to evaluate the effects of different plant extracts, bio-control agents, and chemical nematicides against RKN on Kiwifruit seedlings. The experiment was set up in a Completely Randomized Design with three replications and eight treatments which include the extracts of *Allium sativum* and *Lantana camara*, *Trichoderma viride*, *Pseudomonas fluorescens*, Cartap hydrochloride, Fosthiazate, Inoculated control and Uninoculated control. The results revealed that *Trichoderma viride* proved to be the most effective in reducing the nematode population, displaying a low root gall index of 3.11, a minimal reproductive factor of 0.24, and a high percentage of nematode control at 91.71%. It was also found to be efficient in promoting the growth parameters of Kiwifruit seedlings. Additionally, regression analysis exhibited a significantly positive interaction between root gall index and reproductive factor, while indicating a negative interaction between reproductive factor and growth parameters. Therefore, *T. viride* (@ 20 gm per 2000 cm^3 of soil) should be soil drenched before the seedlings are transplanted into the main field for effective and sustainable management of RKN. Nevertheless, further research is needed to determine the efficacy of *T. viride* in infested roots of Kiwifruit trees in field condition of Kiwifruit orchard.

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Introduction

Kiwifruit is an emerging fruit crop which is popular and suitable to the mid hills and high hills of Nepal at an altitude of 1200 to 2500 masl (Sharma et al., 2020). Though introduced in 1986 AD, commercial kiwifruit cultivation started in Nepal only in 2009 AD (Atreya et al., 2020). It has a tremendous potential for processed value-added products besides fresh fruit, such as jam, jelly, candy, marmalade, wine, juice, etc. which shows huge prospects to increase the production of Kiwifruit in Nepal for uplifting the livelihood of Nepalese famers and commercializing agriculture industry of Nepal (Sharma et al., 2020). While the cultivation area is expanding, the increase in production and productivity has not been entirely satisfactory. The unregulated cultivation of kiwifruit seedlings by Nepalese farmers from unknown sources without adequate phytosanitary measures has led to an escalation in the infestation of root-knot nematodes (RKN) (*Meloidogyne* spp.) in kiwifruit orchards which is considered as a primary contributing factor to the decline in kiwifruit production in Nepal.

It is reported that, more than 40-50 % of Kiwifruit orchard in Nepal including Kiwifruit orchard of Warm Temperate Horticulture Center (WTHC), Kirtipur, Kathmandu is infested with RKN (APR, 2022). Its damage results in poor growth, reduces productive year, quality, and yield, and decreases the resistance of crop against drought and diseases (Subedi et al., 2020). Moreover, they are destructive endoparasite causing average crop losses to 15-20 % annually in the tropical and sub-tropical countries like Nepal (Terefe, 2015; Chhetri, 2019). Kepenekci et al.

(2017) reported that 21-35% annual production loss in Kiwifruit is caused by this notorious pest. In fact, it is difficult to control the soil borne pathogen like RKNs having a wide range of host plants and ability to damage multiple agricultural crops without proper investigation and repeated trials (Muthulakshmi et al., 2010). In addition, there exist a dearth of research and innovation in Nepal to find out the effective, and sustainable control methods against RKN infestation on Kiwifruit.

WTHC, a government-owned fruits' seedlings distributor in Nepal, holds a mandate to produce disease free seedlings. Otherwise, chances of spreading diseases from nurseries to the main field will increase. Given the substantial potential for Kiwifruit to establish itself as a key fruit in Nepal, this study will aid WTHC in identifying an effective control strategy against RKN, enabling the fulfillment of its mandate to provide farmers with healthy seedlings.

Materials and Methods

Research Site

The research was conducted in the Warm Temperate Horticulture Centre (WTHC), Kirtipur, Kathmandu, Nepal located at 27º40'27" N Latitude, 85º17'20" E Longitude and at an altitude of 1303 masl under screenhouse conditions during summer season of 2023 from March to July. WTHC is a government owned fruits seedlings' producer and distributor.

Disease Confirmation and Pathogen Identification

The Kiwifruit trees exhibiting visible symptoms above ground, such as wilting and stunted growth (Hafiza et al., 2016), were chosen from the WTHC fruit orchard. To verify the presence of RKN infestation, the roots of the Kiwifruit were gathered and brought to the Plant Pathology Laboratory at WTHC. The examination revealed the definite presence of root-knots, as depicted in Figure 1.

Juveniles of nematode were extracted from soil following the methods described by Hooper et al. (2005) and Baidya et al. (2023) with some modifications. At first, soil from the vicinity of the Kiwifruit roots was obtained and transported to the laboratory which was then pulverized finely with sterilized hand. A tissue paper was spread inside a plastic sieve placed on an extraction plastic tray. 100 grams of soil sample was spread over the tissue paper and clean tap water was meticulously added from one side of the extraction tray until the soil layer was completely moist. The extraction sets were left undisturbed for 24 hours, allowing the juveniles to migrate from the soil to the extraction tray. The soil-filled plastic sieves were lifted after 24 hours to collect water into the extraction tray. The water with nematodes from the extraction tray was poured into a labelled beaker. The tray was rinsed using a wash bottle, and water was added to the beaker to generate a stock solution, volume of 250 millilitres. After thoroughly stirring, 10 ml of suspension from the stock solution was pipetted out and placed into a petri plate. This small amount of the suspension was examined under a stereomicroscope to aid in identification. The whole set up of the juvenile extraction is shown in the Figure 2. Under the stereomicroscope, juvenile of RKN was observed as shown in the Figure 3.

Figure 1. Sample of Kiwifruit roots showing the formation of root-knots

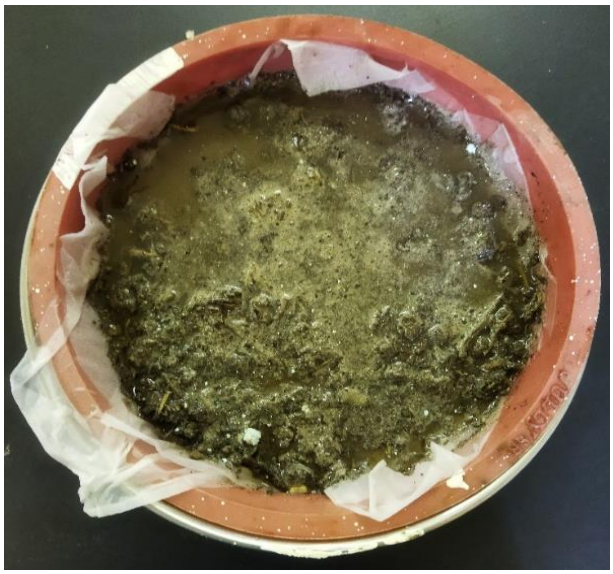

Figure 2. Set up for the extraction of juveniles from the soil using sieve, muslin cloth, and extraction tray

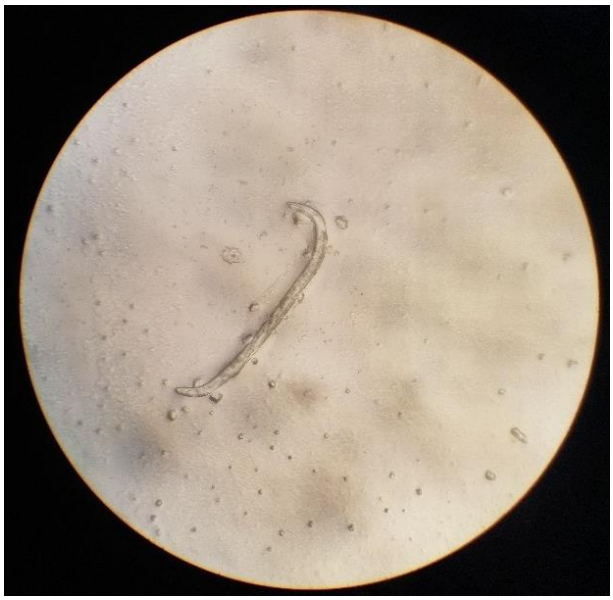

Figure 3. Second stage juvenile (J2) of RKN as observed under stereomicroscope (scale: 10X)

Seedlings Preparation and Transplantation

The seeds were extracted from the Kiwifruit and collected in tissue paper. These seeds were washed to make free from the pulp and the preliminary selection of the seeds were based on their potential viability done by placing the seeds in water for priming for 24 hours. The viable primed seeds were then treated with gibberellic acid @ 500 ppm for 24 hours to promote germination followed by air drying for 24 hours (Lawes & Anderson, 1980). Seedlings were prepared in the germination tray. One-third portion of vermi-compost and coco-peat were mixed with two-third portion of sand. This mixture was filled in a germination tray followed by sowing of sterilized air-dried seeds at the depth of 2 cm. The germination tray was then covered with jute bag and kept in a screen house maintained at the temperature of 30±2 ^{0}C. Watering was done once in two days.

Soil collected from the field was sterilized in an autoclave for 30 min at 15 psi and 120 ^{0}C for the potting mixture, which was prepared by adding sterilized soil mixture with vermicompost in the ratio of 3:1. This mixture of volume 2000 cm^3 was then filled in a plastic pot having inner diameter of 17 cm, where 120 seedlings of the four-leaf stage Kiwifruit seedlings at 60 days after sowing were transplanted @ one plant per pot.

Egg Mass Extraction and Eggs Collection

A heavily knotted roots of the Kiwifruit trees from WTHC, Kirtipur, were used for the extraction of eggs. The technique used for the extraction of eggs from the roots follows the procedure described by Hussey & Barker (1973) and McClure et al. (1973) with some adjustments.

At first, the knotted root sample was cleaned in tap water to remove soil and detached from plant with scissors. 10 gm of sample knotted roots were measured with the help of digital weighing balance which were later chopped into small pieces. The chopped knotted roots were vigorously shaken in magnetic stirrer in 250 ml conical flask containing 1 % sodium hypochlorite solution for 4 min to dissolve the gelatinous matrix of egg sac present in the knotted roots. The resulting suspension of eggs and root debris were poured through 100 and 500-mesh sieves to retain eggs on the 500-mesh sieve. The eggs collected on the 500-mesh sieve were thoroughly washed with distilled water and poured from the sieve into the beaker making a 250 ml solution for the eggs collection. Eggs which escaped through 500-mesh sieve were also recovered by repeated sieving and rinsing. The suspension was stirred, and one ml of aliquot was placed in a nematode counter with the help of a pipette. The eggs were then observed under a stereomicroscope and counted. The counted eggs were kept in refrigerator at 10^0 C for one day to prevent hatching before inoculation.

Inoculation of RKN Eggs

After seven days of seedlings transplantation, 2-3 cm deep holes were made close to each plant with a plastic stick and the plants were inoculated with nematode eggs @ 1500 eggs per pot, except in an uninoculated control (T_8) which was nematode free, using a pipette and plastic syringe. After inoculation, holes around the plants were covered with the adjacent soil.

Experimental Design

The experiment was laid out in a completely randomized design with three replications and eight treatments. There were five pots per treatment in each replication, making 40 pots per replication and 120 as total number of pots used in the research.

Treatments Detail and Methods of Application

Various treatments were evaluated in the nursery condition of Kiwifruit against RKN caused by *Meloidogyne* spp. The treatments were applied after 7 days of the RKN eggs inoculation in their respective doses, shown in the Table 1, to reduce the impact of RKN on kiwifruit seedlings after its infestation. The two botanical extracts, two biological agents, and two chemical nematicides were compared and evaluated for the management of RKN. *Allium sativum* cloves extract and *Lantana camara* leaves extract were used as a botanical agent. The extracts were prepared by grinding the 100 gm of botanicals in the presence of 100 ml of water in an electric blender followed by filtration through muslin cloth and filter paper. The obtained extracts of 100 % concentration were diluted with distilled water to make different concentrations of 5 %, 10 % and 15 %. The laboratory trial of these concentrations showed the highest mortality rate (65 %) of RKN Juveniles at 15 % concentration. In addition, 75 % of eggs were prevented from hatching, thus plant extracts of 15 % concentration were drenched in the soil @ 40 ml per pot following the research conducted by Subedi (2022) on tomato where 40 ml for the 2500 cm^3 volume of soil was used.

The commercial powdered form of biological agents, *Trichoderma viride* and *Pseudomonas fluorescens* were applied directly on soil based on their recommended dose @ 20 gm per 2000 cm^3 of soil. Moreover, soil was treated with synthetic chemical, Cartap hydrochloride in the form of granule @ 5 gm per volume of soil used in each pot which was its recommended dose and another chemical nematicide, Fosthiazate was first diluted @ 1ml in 1 litre of distilled water to make 30 ml of diluted solution which was then drenched on the soil based on its recommended dose of 30 ml per 2000-2500 cm^3 of soil.

In inoculated control, nematode eggs were inoculated in the soil, but no treatments were used for the control. In uninoculated control, neither nematode eggs were inoculated, nor any treatments were used for the RKN control, i.e., seedlings were grown in a sterilized soil. During the time of treatments application, pots with inoculated control and uninoculated control were soil drenched with 100 ml of distilled water to make uniform application of the treatment.

Observation Parameters and Data Collection

After two and half months of nematode eggs inoculation, sampled pots from the screenhouse were cautiously carried to the Plant Pathology laboratory of WTHC, Kirtipur, where kiwifruit seedlings were carefully uprooted, washed with tap water, and different observation parameters were carried out.

Gall Formation Quantity at Roots

All the soil debris attached with the roots were carefully removed by washing gently with tap water and symptomatic assessment of root gall intensity was carried out based on a scale (0-10) given by Bridge & Page (1980). The different scale based on the level of infection by RKN is depicted in Table 2.

Table 1. Treatments details used for management of RKN (Meloidogyne spp.) at WTHC, Kirtipur, Kathmandu, Nepal, 2023

S.N.	Name of treatments	Symbolic representation	Application dose per pot
1.	Allium sativum extract	T_1	40 ml solution of 15 % conc^n
2.	Lantana Camara extract	T_2	40 ml solution of 15% conc^n
3.	Trichoderma viride	T_3	20 gm powder
4.	Pseudomonas fluorescens	T_4	20 gm powder
5.	Cartap hydrochloride 4% GR	T_5	5 gm granules
6.	Fosthiazate 20% EW	T_6	30 ml of solution
7.	Inoculated control	T_7	100 ml of distilled water
8.	Uninoculated control	T_8	100 ml of distilled water

Table 2. A scale for determining root gall index caused by *Meloidogyne* spp. based on a Bridge & Page (1980) explanation

Root gall index (0-10)	Explanation of rating
0	Healthy plant root system without knot formation
1	Very few small knots, difficult to find and can only be detected on close inspection
2	Small knots only, but clearly visible, main roots clean
3	Some larger knots visible, main roots free
4	Larger knots predominate but main roots free
5	50 % of roots are infested. Knotting on parts of main roots, reduced root system
6	More knotting on main roots
7	Majority of main roots knotted
8	All main roots knotted. Few clean roots visible
9	All roots severely knotted and plant usually dying
10	All roots severely knotted. No root system. Plant usually dead.

All secondary roots were separated from primary root by carefully cutting with scissors. Knots on each primary and secondary root of each plant of each treatment were counted with naked eyes. Mean number of galls per plant per treatment was calculated. The same roots were used for the extraction of eggs.

Egg count

The same roots which were used for the assessment of gall formation index were used for the extraction of eggs. 10 gm sample of Knotted portion of roots were chopped with the help of scissors and were subjected to the procedure as described in section 2.4. The eggs thus obtained were counted in the nematode counter with the help of stereomicroscope. Total number of eggs present in the root system was calculated by multiplying the eggs present in 10 gm sampled knotted roots to the total weight of knots present in a root system.

Juvenile count

Total volume of soil present in each pot was used for the juvenile extraction. The whole extraction process of juveniles follows the description as given in section 3.2 based on Hooper et al. (2005) and Baidya et al. (2023) with some modifications. After stock solution was prepared as mentioned in the section 3.2, 10 ml of suspension was pipetted out and placed into counting plates for the juvenile count. The total number of juveniles present in 10 ml of suspension was counted, and using this number, the total number of nematodes present in the 250 ml of the stock solution (corresponding to 100 grams of soil) was determined.

Reproductive factor (Rf)

After counting of nematode eggs present in the knotted roots of Kiwifruit seedlings and juveniles present in the soil of each pot, final population of root knot nematode was computed by adding mean number of eggs per plant and mean number of juveniles per 100 gm soil in each treatment. Rf of *Meloidogyne* spp. was calculated by dividing the final population (Rf) with the initial population (Pi). Thus, Rf is number of nematodes (eggs and juveniles) produced from one egg, inoculated at the beginning of the experiment.

$$\text{Reproductive factor } (R_f) = \frac{\text{Final population (Pf)}}{\text{Initial Population (Pi)}}$$

It is believed that when Rf (= Pf/Pi) is ≥ 1, nematodes can grow and develop a population. On the other hand, when the ratio is ≤ 1, nematodes neither grow nor develop a population in agro-ecosystem, hence cannot impose a damage to the culture plants (Ferris & Noling, 1987).

Plant height and shoot weight

After the Kiwifruit seedlings were carefully uprooted, they underwent a thorough washing with tap water at the Plant Pathology Laboratory of WTHC. Using scissors, the above-ground section was separated from the roots. The length of the above-ground portion was measured using a 30 cm scale to ascertain the plant height. From each replication, 3 out of 5 randomly selected plants per treatment were chosen for sample measurement. The same above ground portion was used to determine the shoot weight which was measured using digital weighing balance.

Root length and root biomass

The roots which were separated from the shoot portion were cleaned gently using tap water and left for some time so that the excess water present in the roots evaporates. Root length was measured on primary root, using a 30 cm length scale. 3 sample plants per treatment per replication were selected to measure root length. The same roots were used the measurement of root biomass using digital weighing balance.

Percentage nematode control

It was calculated using the formula given by Davis et al. (2009) with some modifications.

$$\text{Percentage Nematode Control} = \frac{A-B}{A} \times 100\ \%$$

Where, A = Nematode population at inoculated control
B = Nematode population at treatment

Statistical Analysis

The gathered data was organized and tabulated into MS-Excel. Analysis of variance was conducted for all the parameters utilizing the statistical software R-Studio, version: 2023.06.2+561. In addition, the Duncan Multiple Range Test (DMRT) was employed for distinguishing means using the same version of R-studio. Regression analysis was performed using XLSTAT.

Results

Root Gall Index and Number of Root Galls Per Plant

A significant variation was observed between various treatments (at p≤0.001) in terms of both root gall index and number of root galls per plant as shown in the table 3. *Trichoderma viride* had the lowest root gall index (3.11) and number of root galls per plant (18.00) followed by Fosthiazate with root gall index (4.00) and number of root galls per plant (34.00), while inoculated control had the highest root gall index (7.44) and number of root galls per plant (132.00).

Egg Count, Juvenile Count, and Reproductive Factor (Rf)

There was significant difference between different treatments at 0.1% level of significance in terms of egg count, juvenile count and reproductive factor as shown in the table 4. The lowest value of egg count and juvenile count was obtained in *Trichoderma viride* (333.33 and 32.67) followed by chemical Fosthiazate (533.33 and 70.67), and *Pseudomonas fluorescens* (763.33 and 109.33) respectively. Contrarily, the highest egg count and juvenile count was obtained in inoculated control (4120.00 and 299.00) followed by *Allium sativum* extract (1516.67 and 167.00) and *Lantana camara* extract (1333.33 and 140.33) respectively.

Trichoderma viride had the lowest reproductive factor (0.24) which was ahead of Fosthiazate (0.402) and *Pseudomonas fluorescens* (0.58) whereas the highest reproductive factor was obtained in Inoculated control (2.95) followed by *Allium sativum* extract (1.12).

Effect on Plant Height and Shoot Weight

Uninoculated control had the highest plant height (6.00 cm) followed by *Trichoderma viride* (4.88 cm), whilst lowest value was observed in inoculated control (2.93 cm) as shown in the table 5. In addition, the highest shoot weight was obtained in uninoculated control (3.70 gm) followed by *Trichoderma viride* (2.80 gm) and *Pseudomonas fluorescens* (1.99 gm). Lowest shoot weight was obtained in inoculated control (1.40 gm) followed by Cartab hydrochloride (1.52 gm).

Table 3. Root gall formation evaluation of different plant extracts, biocontrol agents, and chemical pesticides against RKN (*Meloidogyne* spp.) on Kiwifruit seedlings at WTHC, Nepal

S.N.	Treatments	Root Gall Index (0-10)	Number of galls per plant
1	*Allium sativum* extract	5.44^{bc}	79.67^{b}
2	*Lantana camara* extract	4.89^{cd}	58.33^{c}
3	*Trichoderma viride*	3.11^{f}	18.00^{e}
4	*Pseudomonas fluorescens*	4.45^{de}	49.33^{c}
5	Cartap Hydrochloride 4 % GR	5.78^{b}	76.67^{b}
6	Fosthiazate 20 % EW	4.00^{e}	34.00^{d}
7	Inoculated Control	7.44^{a}	132.00^{a}
	SEm (±)	0.37	4.51
	F-test	***	***
	CV (%)	9.05	8.62
	LSD (0.05)	0.79	9.67
	Grand Mean	5.01	64

Means followed by common letter(s) within a column do not differ significantly at ≤5 % level of significance by DMRT; LSD = Least significant difference; significance codes ***at p≤0.001; **at p≤0.01; *at p≤0.05; SEm= Standard error of mean, CV = Coefficient of variation

Table 4. Effect of different plant extracts, biocontrol agents, and chemical pesticides on the reproductive performance of RKN (*Meloidogyne* spp.) on Kiwifruit seedlings at WTHC, Kirtipur, Kathmandu

S.N.	Treatments	Final Population (No.)		Reproductive Factor
		Eggs	Juveniles	(Rf)
1	Allium sativum extract	1516.67^{b}	167.00^{b}	1.12^{b}
2	Lantana camara extract	1333.33^{c}	140.33^{c}	0.98^{c}
3	Trichoderma viride	333.33^{g}	32.67^{f}	0.24^{g}
4	Pseudomonas fluorescens	763.33^{e}	109.33^{d}	0.58^{e}
5	Cartap Hydrochloride 4 % GR	1133.33^{d}	156.67^{b}	0.86^{d}
6	Fosthiazate 20 % EW	533.33^{f}	70.67^{e}	0.40^{f}
7	Inoculated Control	4120.00^{a}	299.00^{a}	2.95^{a}
	SEm (±)	72.97	5.94	0.05
	F-test	***	***	***
	CV (%)	6.43	5.22	5.80
	LSD (0.05)	156.50	12.74	0.10
	Grand mean	1390.48	139.38	1.02

Means followed by common letter(s) within a column do not differ significantly at ≤5 % level of significance by DMRT; LSD = Least significant difference; significance codes ***at p≤0.001; **at p≤0.01; *at p≤0.05; Sem= Standard error of mean, CV = Coefficient of variation

Table 5. Evaluation of different plant extracts, biocontrol agents, and chemical pesticides on the growth parameters of Kiwifruit seedlings caused by RKN (*Meloidogyne* spp.) at WTHC, Kirtipur, Kathmandu, 2023

S.N.	Treatments	Plant height (cm)	Shoot weight (gram)	Root length (cm)	Root weight (gram)
1	Allium sativum extract	4.11^{c}	1.88^{c}	6.74^{bc}	1.25^{c}
2	Lantana camara extract	3.77^{c}	1.59^{c}	4.84^{c}	1.03^{c}
3	Trichoderma viride	4.88^{b}	2.80^{b}	8.29^{ab}	1.87^{b}
4	Pseudomonas fluorescens	3.71^{c}	1.99^{c}	7.31^{b}	1.39^{c}
5	Cartap Hydrochloride 4 % GR	4.01^{c}	1.62^{c}	6.28^{bc}	1.13^{c}
6	Fosthiazate 20 % EW	3.65^{c}	1.52^{c}	6.99^{bc}	1.22^{c}
7	Inoculated Control	2.93^{d}	1.40^{c}	4.79^{c}	1.02^{c}
8	Un-inoculated Control	6.00^{a}	3.70^{a}	9.64^{a}	2.57^{a}
	SEm (±)	0.30	0.31	1.004	0.18
	F-test	***	***	**	***
	CV (%)	8.90	18.13	17.92	15.75
	LSD (0.05)	0.63	0.65	2.12	0.39
	Grand mean	4.13	2.06	6.85	1.43

Means followed by common letter(s) within a column do not differ significantly at ≤5 % level of significance by DMRT; LSD = Least significant difference; significance codes ***at p≤0.001; **at p≤0.01; *at p≤0.05; Sem= Standard error of mean, CV = Coefficient of variation

Table 6. Effect of treatments on percentage nematode control of Kiwifruit seedlings at WTHC, Kirtipur, Kathmandu, 2023

S.N.	Treatments	Percentage nematode control (%)
1	Allium sativum extract	61.86^{f}
2	Lantana camara extract	66.66^{e}
3	Trichoderma viride	91.71^{a}
4	Pseudomonas fluorescens	80.25^{c}
5	Cartap Hydrochloride 4 % GR	70.80^{d}
6	Fosthiazate 20 % EW	86.33^{b}
	SEm (±)	1.69
	F-test	***
	CV (%)	2.72
	LSD (0.05)	3.69
	Grand mean	76.27

Means followed by common letter(s) within a column do not differ significantly at ≤5 % level of significance by DMRT; LSD = Least significant difference; significance codes ***at p≤0.001; **at p≤0.01; *at p≤0.05; SEm= Standard error of mean, CV = Coefficient of variation

Effect on Root Length and Root Biomass

A significant difference was observed between treatments in terms of root length (p≤0.01) and root weight (p≤0.001) as shown in the table 5. Uninoculated control had the highest root length (9.64 cm) followed by *Trichoderma viride* (8.29 cm). Contrarily, the lowest root length was obtained in inoculated control (4.79) followed by *Lantana camara* extract (4.83 cm) and Cartap hydrochloride (6.28 cm).

In addition, Uninoculated control had the highest root biomass (2.57 gm) followed by *Trichoderma viride* (1.87 gm) and *Pseudomonas fluorescens* (1.39 gm). In contrast, Inoculated control had the lowest root biomass (1.02 gm) which was statistically at par with other treatments except *T. viride* and uninoculated control.

Percentage Nematode Control

Percentage nematode control was significantly higher in *Trichoderma viride* with 91.70 % control followed by chemical Fosthiozate with 86.33 % control. On the contrary, the lowest percentage of nematode control was obtained with *Allium sativum* extract (61.86 %) followed by *Lantana camara* extract (66.66 %).

Interaction Between Reproductive Factor (Rf) and Root Gall Index (GI)

A significantly positive relationship between root gall index and Rf was observed as shown in the figure 4. Linear regression equation revealed that if there was a unit increase in Rf, root gall index would have been increased by 1.4038 times. According to the coefficient of determination, contribution of Rf for increment in the root gall index was found 77.06 %.

Interaction between Reproductive Factor (Rf) and Shoot Weight, and Root Biomass

A significantly negative relationship between Rf and growth parameters like shoot weight and root biomass was observed as shown in the figure 5 and figure 6 respectively. According to the linear regression equation, if there was a unit increase in Rf, shoot weight and root biomass would have been decreased by 0.2892 and 0.1965 times respectively. Moreover, according to the coefficient of determination, contribution of Rf for reduction in shoot weight and root biomass in Kiwifruit seedlings was 19.74 % and 26.61 % respectively.

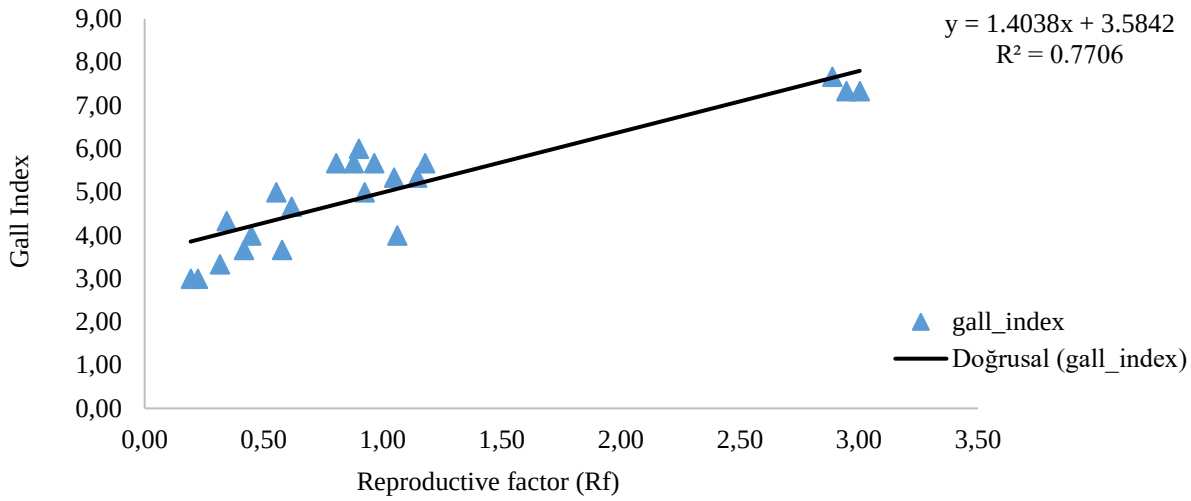

Figure 4. Regression between root gall index and Rf in Kiwifruit at WTHC, Kirtipur, Kathmandu

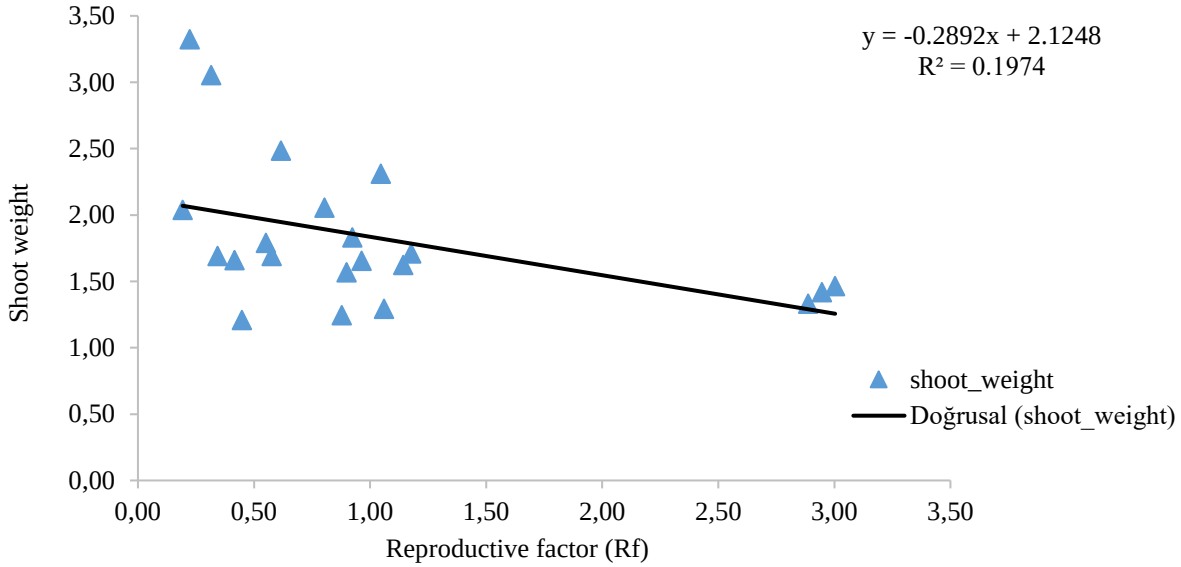

Figure 5. Regression between Rf and shoot weight on Kiwifruit at WTHC, Kirtipur, Kathmandu

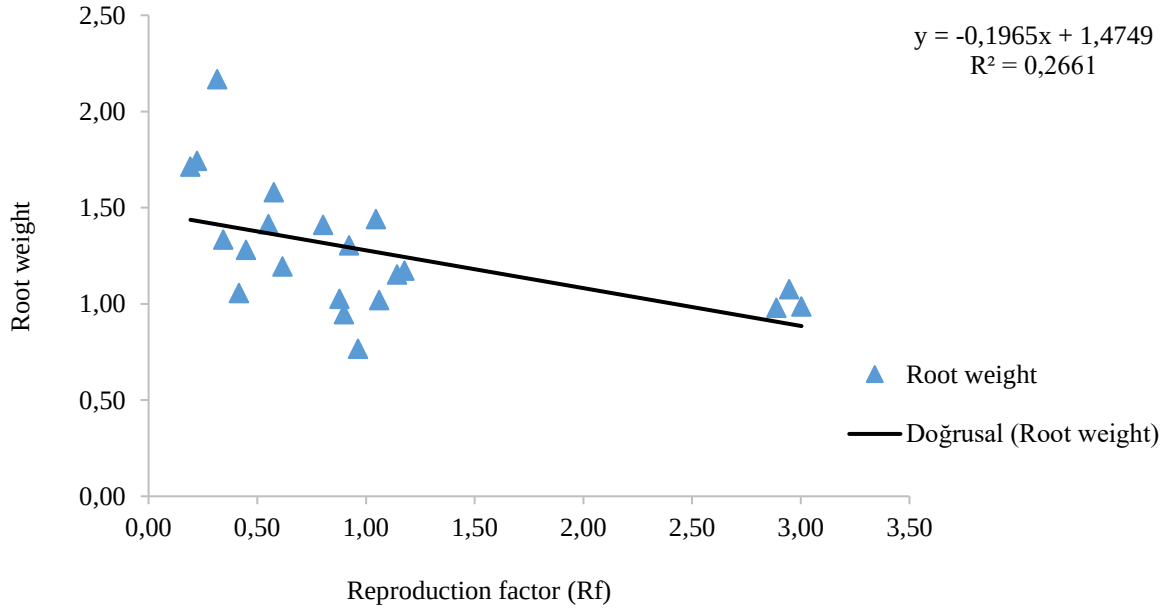

Figure 6. Regression between Rf and root weight on Kiwifruit at WTHC, Kirtipur, Kathmandu

Discussions

Root Gall Index and Number of Root Galls per Plant

Our investigation revealed a noteworthy distinction among various treatments tested against *Meloidogyne* spp. on Kiwifruit seedlings, particularly concerning the root gall index and the quantity of root galls per plant. Notably, the lowest values for both root gall index and the number of root galls per plant were observed in the case of *Trichoderma viride*. This finding aligns with the results of a similar study conducted by Subedi (2022) on tomatoes, where *Trichoderma viride* demonstrated a significant reduction in the gall index (4.00) compared to the control (8.33). Baños et al. (2017) also discovered that *Trichoderma viride* led to a 53.5% reduction in gall index caused by *Meloidogyne* spp. Furthermore, Baidya et al. (2023) found that *Trichoderma viride* significantly decreased both the gall index (3.43) and the number of root galls per plant (88.86) in tomato, lending further support to our own findings.

In addition, Shamalie et al. (2012) noted that the application of *T. viride* in soil led to only 13.32% of root gall formation in *Centella asiatica*. The formation of galls on the roots and the invasion of root tissue by these nematodes deprive plants of vital nutrients (Terefe, 2015), resulting in stunted growth and heightened vulnerability to mild stressors. *Pseudomonas fluorescens* also demonstrated a significant decrease in both the gall index and the quantity of gall formation. Abd-El-Khair et al. (2019) reported a 57% reduction in gall formation on cowpeas due to *Pseudomonas fluorescens*.

Egg Count, Juvenile Count, and Reproductive Factor (Rf)

Amid the various treatments, *Trichoderma viride* emerged as the most effective in controlling the final nematode population, aligning with Subedi (2022) study on RKN in tomato plants, where *Trichoderma viride* displayed the lowest egg count (6720) and juvenile count (255). Baidya et al. (2023) also noted a significant reduction in the nematode population due to *Trichoderma* spp., with a reproductive factor of 0.45, slightly exceeding the findings of the current study. A reproductive factor less than one indicates that nematodes neither grow nor develop a population in the soil that could harm the crops of interest (Ferris & Noling, 1987). Elad et al. (1982) emphasized the production of antibiotics and extracellular lytic enzymes by *Trichoderma* spp. as effective tools against RKN. Additionally, Santos et al. (1992) reported that *Trichoderma* spp. acted as effective egg parasites of *Meloidogyne* spp. by growing on the surface of RKN egg masses and penetrating the eggshell. Destruction of the egg masses subsequently led to a significant reduction in the number of infective juveniles, ultimately decreasing the final nematode population and reproductive factor. Haran et al. (1996) highlighted the role played by enzymes like chitinase, glucanases, and proteases produced by *Trichoderma* spp. in parasitism.

Chemicals such as Fosthiazate and Cartap hydrochloride, along with the biocontrol agent *Pseudomonas fluorescens*, were also found effective against RKN in Kiwifruit seedlings. Hashem & Abo-Elyousr (2011) reported that *P. fluorescens* caused approximately 45% mortality in J2 of RKN in tomato. Moreover, Abd-El-Khair et al. (2019) found that *Pseudomonas fluorescens* significantly reduced the number of egg masses per plant by 60% and J2 in the soil by 76%.

Furthermore, the leaf extract of *Lantana camara* exhibited potential in reducing the reproductive factor, whereas *Allium sativum* was deemed ineffective, as the RF was determined to be 1.12, indicating the potential for RKN multiplication within the agro-ecosystem. The diverse chemical composition present in *L. camara*, including compounds such as 11-oxo triterpenic acid, lantanolic acid, lantoic acid, pomolic acid, and cimarin, may contribute to the reduction of the nematode population (Chitwood, 2002; Ntalli & Caboni, 2012). Similar findings were also reported in a study conducted by Abrar et al. (2020), where the leaf extract of *Lantana camara* significantly reduced the RF to 0.5306. Additionally, Feyisa et al. (2015) reported highly effective results from the use of *Lantana camara* extracts, with an RF as low as 0.26, differing from the current study's results.

Growth Parameters (Plant Height, Shoot Weight, Root Length, and Root Biomass)

A noticeable distinction at the 0.1% level of significance was observed among the various treatments for plant height, shoot weight, and root biomass, while treatments demonstrated significant variations at the 1% level of significance for root length. Notably, the uninoculated control exhibited the highest values for all growth parameters, including plant height (6.00 cm), shoot weight (3.69 gm), root length (9.63 cm), and root biomass (2.56 gm). Conversely, the inoculated control displayed the lowest values for all growth parameters, indicating that the roots of Kiwifruit seedlings may have been deprived of water and nutrients uptake from the soil due to a severe infestation of *Meloidogyne* spp., consequently hampering the plants' growth performance.

Among the applied control measures, *Trichoderma viride* proved to be an effective treatment, consistent with the findings of Shamalie et al. (2012), which revealed that the application of *Trichoderma viride* in the soil led to an increase in stalk length to 14.07 cm, root length to 2.97 cm, and top fresh weight to 0.9 gm compared to the untreated control. Similarly, Subedi (2022) discovered that the application of *Trichoderma viride* in the soil resulted in a dry root weight of 40.75 gm, surpassing the results of the untreated control. Baidya et al. (2023) also reported similar findings regarding the impact of *Trichoderma viride* on tomato plants, where the fresh root weight was significantly higher (5.51 gm) compared to the control (3.65 gm). According to Hermosa et al. (2012), *Trichoderma* spp. can indirectly interact with roots, thereby enhancing the potential for plant growth. Alongside *T. viride*, *Pseudomonas fluorescens* also exhibited effectiveness against RKN. Abd-El-Khair et al. (2019) observed that the introduction of *Pseudomonas fluorescens* increased the fresh shoot weight and root weight by 35% and 19% respectively compared to the inoculated control in cowpeas, supporting the findings of our study.

Percentage Nematode Control

It was noted that the application of different treatments significantly aided in controlling the nematode population in comparison to inoculated control. Soil drenching with *Trichoderma viride* emerged as an effective measure, leading to a 91.70% control of RKN on Kiwifruit seedlings. Given that chitin constitutes a major component of the nematode eggshell, the nematophagous egg-parasitic fungus *Trichoderma viride* can infiltrate the eggs, thereby managing the nematode population (Druzhinina et al., 2018; Kubicek et al., 2019). The biocontrol agent *Pseudomonas fluorescens* was also observed to be effective, controlling the nematode population by 80.25%, surpassing the results obtained by Abd-El-Khair et al. (2019) in cowpeas, where a 69.8% nematode control was achieved through the application of *Pseudomonas fluorescens*. The rhizobacteria under study might employ various mechanisms such as the production of antibiotics, enzymes, and toxins against plant-parasitic nematodes, leading to the reduction in nematode populations (Siddiqui et al., 2006).

Allium sativum, when compared with other treatments, was found to be less effective, resulting in a 61.86% nematode control rate. The release of various sulfur compounds from garlic clove extract could be implicated in nematode control. Leaf extracts of *Lantana camara* were also found to be effective in controlling the nematode population (66.66%). Similar findings were observed in a study conducted by Taye et al. (2012) in Ethiopia, where a 59% control rate on the final nematode population was observed in tomato roots with the use of *Lantana camara* leaf extracts. Similarly, in a study by Srivastava et al. (2006), *L. camara* was found to be effective against *M. incognita*, resulting in a mortality rate of 85-90%. Ntalli & Caboni (2012) reported that the presence of chemicals like 11-oxo triterpenic acid, lantanolic acid, lantoic acid, pomolic acid, camarin, lanatacin, and ursolic acid accounts for the antagonistic effect of L. camara against RKN.

Conclusion

The findings of the present study indicated that *Trichoderma viride* effectively combated RKN, leading to a notable reduction in the formation of root galls on Kiwifruit seedlings. The treatment with *Trichoderma viride* resulted in the lowest counts of eggs and juveniles, along with a minimized reproductive factor, thereby achieving the highest percentage of nematode control. The growth parameters of Kiwifruit seedlings also displayed improved performance under the *Trichoderma viride* treatment, coming in second only to the uninoculated control group.

Moreover, a significant positive correlation was identified between the Rf and the root gall index, while a negative correlation was observed between the Rf and the growth parameters of Kiwifruit seedlings. Consequently, the application of *T. viride* is recommended to mitigate the infestation of RKN on Kiwifruit seedlings.

Limitations of the Study

This research is limited to a single season only. In addition, this research was carried out in screenhouse condition; hence, further research is advised to implement the same treatments in a Kiwifruit orchard, aiming to ascertain their effectiveness under field conditions.

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Declarations

Author contribution statement

Kapil Simkhada: Conceived and designed the research, performed the research, analyzed, and interpreted data, wrote the paper.

Srijana Bhandari: Design the research, contributed to the materials and methods.

Chiranjivi Sharma: Analysis and interpretation of data, contributed to paper writing.

Data availability statement

Data will be made easily available on request.

Declaration of interest statement

The authors declare no conflict of interest in publishing this scientific paper.

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Electrochemical Non-Enzymatic Glucose Sensing Platform Based on Vanadium Pentoxide Film-Modified Screen Printed Gold Electrode

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ABSTRACT

A screen printed gold electrode (SPGE) served as the foundation for directly depositing Vanadium pentoxide (V_2O_5), crafting an enzyme-free glucose sensor. Through cyclic voltammetry in an alkaline setting, the sensor's ability to drive glucose oxidation was explored. Utilizing V_2O_5 as an electrocatalyst, this non-enzymatic sensor exhibited an expansive linear detection range (1 mM–10 mM) and an impressively low detection limit of 0.9 µM. These results underscored V_2O_5's robust electrocatalytic process in facilitating glucose oxidation within alkaline solutions, unaffected notably by substances like ascorbic acid, fructose and maltose. This investigation highlights a direct and efficient method for glucose detection without reliance on enzymes.

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Introduction

D-Glucose (DGLC) acts as the main energy source for the human body and plays a role in forming metabolic intermediates. However, deviations in DGLC levels in the bloodstream are associated with diabetes mellitus (Rahman et al., 2019). Regardless of socioeconomic status, diabetes has notably surged in many middle-income countries across the globe. Although chronic, diabetes can lead to severe complications and symptoms such as cardiac issues, blindness, ocular problems, peripheral vascular diseases, strokes and kidney failure. The body typically maintains blood DGLC levels within a range of 70–120 $mgdL^{-1}$. Diabetic individuals often exhibit significantly elevated DGLC levels due to their inability to regulate their sugar levels (Razak et al., 2016). Extensive research in diabetes management shows that closely watching blood glucose levels can postpone the development and advancement of complications associated with diabetes (Dong et al., 2021).

Accurate measurement of glucose holds significance in diverse sectors such as the food industry, fuel cells, environmental studies and pharmaceuticals. Given the myriad applications of glucose, multiple detection methods have been employed to quantify it (Dhara and Mahapatra, 2018). Various conventional approaches, including chromatographic (Filip et al., 2016), spectrophotometric (Chitra et al., 2017), chemiluminescence (Wu et al., 2014) and electrochemical methods (Maduraiveeran et al., 2018) have been devised to analyze glucose in real samples. Among these techniques, electrochemical biosensors stand out as highly promising tools and have garnered attention as point-of-care (POC) devices (Phetsang et al., 2019). Electrochemical sensors set the industry standard for glucose detection due to their attributes: low detection limits, heightened reliability, swift response times, operational simplicity and significantly lower costs compared to sensors using alternative detection mechanisms (Wei et al., 2020).

Amperometric electrochemical glucose biosensors relying on the enzyme glucose oxidase have been extensively studied for detecting glucose levels in food and blood, aiming for enhanced electrocatalytic responses and heightened sensitivity (Faisal et al., 2010). However, immobilized enzyme sensors possess certain drawbacks, including instability triggered by intrusive chemicals and alterations in sample pH and temperature. To tackle these

constraints, a different approach has come to light: direct electrochemical oxidation of glucose using nonenzymatic glucose sensors (Toghill and Compton, 2010).

Functionalized nanomaterials serve various roles, functioning as catalysts, immobilization platforms or electro-optical labels, significantly boosting detection sensitivity and specificity (Rathod et al., 2010). The advent of nonenzymatic electrodes, relying on the direct electro-oxidation of glucose, marks the potential for the fourth generation of glucose sensors. These sensors are crafted by integrating nanostructured metals (Bai et al., 2010) or metal oxides (Zhang et al., 2012) onto the electrode surface, displaying markedly enhanced electrocatalytic activity towards glucose, compared to enzyme-based counterparts.

Recently, the scientific community has shifted its focus towards metal oxides, garnering significant attention as promising materials for glucose detection. This interest is primarily attributed to their notably lower costs and enhanced resistance to poisoning and fouling (Zhu et al., 2016). Particularly, NiO (Franceschini and Taurino, 2022), CuO (Ashok et al., 2019) and their composites (Wei et al., 2021) have emerged as prominent candidates, often featuring surface nanostructuring. Additionally, ongoing investigations are exploring oxides such as Co_3O_4, FeO_x and MnO_x (Sattarahmady and Heli, 2012), (Si et al., 2013), (Raza and Ahmad, 2018).

Among the transition series, vanadium and its oxides (VO_x) stand out as incredibly diverse and intriguing catalytic systems. Their applications range from synthesizing vital chemicals (Wachs, 2013) to reducing environmental pollutants (Monfort and Petriskova, 2021) and more recently, being employed in neuromorphic and switching devices (Li et al., 2022). The complexity of their chemistry can be attributed to two primary factors: the multitude of oxidation states in which vanadium can exist (ranging from V^{2+} to V^{5+}) and the varied coordination geometry of oxygen ions. Additionally, these oxides possess a noteworthy ability to create mixed-valence oxides through the inclusion of oxygen vacancy defects (Berenguer et al., 2017). These defects have been found to enhance the adsorption of specific reactants, ultimately catalyzing numerous oxidation or reduction reactions (Kämper et al., 2000).

In disease monitoring applications, screen-printed electrodes (SPEs) hold an edge over other electrode types due to their adaptability for modification, diverse material composition, varied geometries, compact sizes, affordability, simplicity and portability. Therefore, the combination of screen-printing technology with inexpensive nanomaterials holds great promise for crafting biosensing devices. Such devices can operate with minimal sample volumes while showcasing excellent selectivity and sensitivity in their responses (Chu et al., 2017).

This study involves modifying a screen-printed gold electrode through electrochemical deposition of V_2O_5 nanoparticles, followed by activation in an alkaline medium to create SPGE/V_2O_5. The aim was to establish a straightforward sensing platform utilizing the modified electrode as a glucose biosensor. This research work illustrated the creation, classification and establishment of a D-glucose sensor utilizing SPGE/V_2O_5 using cyclic voltammetry as the method of choice.

Experimental

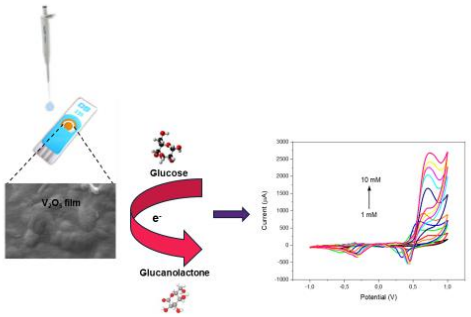
Materials

The substances employed in this research, including vanadium pentoxide, hydrogen peroxide, sodium hydroxide, glucose, ascorbic acid, fructose and maltose were sourced from Sigma-Aldrich Corp. (USA) and met analytical grade standards. Alkaline solutions were prepared using NaOH pellets and all solutions were made using deionized water. The tests were conducted under typical room temperature conditions. Screen printed gold electrodes (SPGE) were obtained from Metrohm DropSens S.L. (Spain).

Construction of the V_2O_5 Non-enzymatic Glucose Sensor

The V_2O_5 film was electrochemically deposited onto the SPGE. To dissolve 0.18 g of V_2O_5 powder, a solution was prepared by vigorously stirring it with 10 mL of deionized water and 0.9 mL of 30% hydrogen peroxide for 1 hour at room temperature. Twenty microliters (20 µL) of this electrolyte solution was applied onto the SPGE. Employing a pulsed electrodeposition technique, initially, the electrode potential was set at 0 V for 20 seconds to achieve equilibrium. Subsequently, it was adjusted to -2.0 V for 10 seconds to aid in the electrophoretic deposition of V_2O_5. This cycle was iterated 60 times (Uchaker et al., 2014).

Glucose solutions ranging from 1 mM to 10 mM were prepared in a 0.1 M NaOH solution. As the glucose concentration increased, anodic peak current rose notably at around +0.7 V due to the interaction between glucose and V_2O_5 in an alkaline solution. To utilize the V_2O_5-based sensor, 20 µL of a known glucose solution in 0.1 M NaOH was drop-casted. A 10-second resting period allowed hydroxide ions to initiate V_2O_5 oxidation. Subsequently, cyclic voltammetry (CV) was performed between -1.0 and +1.0 V. The mechanism of the glucose sensor is proposed in Scheme 1.



Scheme 1. Mechanism of SPGE/V_2O_5 sensor for glucose detection

Instrumentation

Electrochemical evaluations were performed with a Metrohm Dropsens µStat-i potentiostat/galvanostat, employing screen-printed gold electrodes obtained from Metrohm DropSens (Spain). These electrodes comprised a 3-electrode electrochemical cell, including gold-based working and counter electrodes, a quasi-reference

electrode made of silver and electrical contacts. The working electrode had a diameter of 4 mm. A DRP-DSC connector facilitated the connection between the screen-printed electrodes and the potentiostat. CV experiments were executed at a scan rate of 100 mVs^{-1} within the potential range of -1.0 to +1.0 V to evaluate the electrochemical behavior of each sample. For morphological analysis of the V_2O_5-modified electrodes, a LS10 scanning electron microscope (SEM) (Zeiss Evo, Carl Zeiss NTS, Germany) was utilized, outfitted with an energy-dispersive X-ray spectroscopy (EDX) analysis detector (Zeiss Evo). The structural analysis was conducted utilizing a D8 Advance X-ray Diffractometer (XRD) (Bruker, Germany), operating within the 2θ range of 10-60° with a scan rate of 2°/min.

Results and Discussion

SEM images of bare and V_2O_5 modified SPGE are presented in Fig 1a and 1b, respectively. As shown in Fig. 1a, bare SPGE has a porous structure whereas the V_2O_5 SEM image depicts a rough surface marked by prominent globular structures as indicated in Fig 1b. Figure 1c displays the XRD pattern of V_2O_5 film. The XRD analysis reveals a preferential orientation in the V_2O_5 thin film, aligning with the (001) plane at a 2Θ angle of 20.33. This orientation indicates a single phase consistent with the diffraction data JCDPS file No. 41-1426, confirming the orthorhombic structure of V_2O_5 (Berouaken et al., 2022).

Elemental analysis through EDX and mapping of elements verified the chemical composition of V_2O_5, confirming the presence of vanadium (V) and oxygen (O) elements. These elements were distinctly visible in the EDX spectrum of V_2O_5 (Fig. 1d). The amounts of vanadium (V) and oxygen (O) elements were found to be 17.54% and 82.46%, respectively according to EDX analysis. The homogeneous dispersion of elements V and O on the V_2O_5 electrode's surface is illustrated in Fig. 1(e-g) through elemental mapping images, where green and blue colors symbolize each respective element.

CV was employed to assess the electron transfer behavior exhibited by different modified electrodes including the bare SPGE and V_2O_5-modified SPGE electrodes, both before and after the incorporation of glucose into a 0.1 M NaOH solution. The experiment encompassed a potential range from -1.0 to +1.0 V, with a scan rate set at 100 mV s^{-1} and the resulting CVs are depicted in Fig. 2. Notably, the unmodified SPGE electrode displayed no discernible redox peaks within the assessed potential range. In contrast, all modified electrodes exhibited well-defined redox response peaks, indicating distinct interfacial structures that significantly influenced the observed electrochemical responses (Phetsang et al., 2019).

In the absence of glucose, the cyclic voltammogram (CV) exhibited a pair of anodic and cathodic peaks centered around +0.6 and -0.3 V, attributed to the oxidation/reduction reactions of vanadium species. This observation confirmed the effective activation of the electrode for sensing purposes. Upon the addition of glucose to the electrolyte solution, there was an increase in the anodic peak current linked to the augmentation of vanadium species. Additionally, a slight shift to higher potentials was noted, indicating the electrocatalytic mechanism of glucose oxidation facilitated by V_2O_5. In the proposed electrocatalytic reaction mechanism elucidating D-glucose with V_2O_5 through the I–V performance, the electro-oxidation of D-glucose resulted in its transformation into D-gluconolactone, which then converted into D-gluconic acid and hydrogen peroxide. Subsequently, H_2O_2 was converted into oxygen and protons, liberating two electrons. These released electrons were reflected in the current–voltage curve, enabling the detection of D-glucose (Rinaldi and Carballo, 2016).

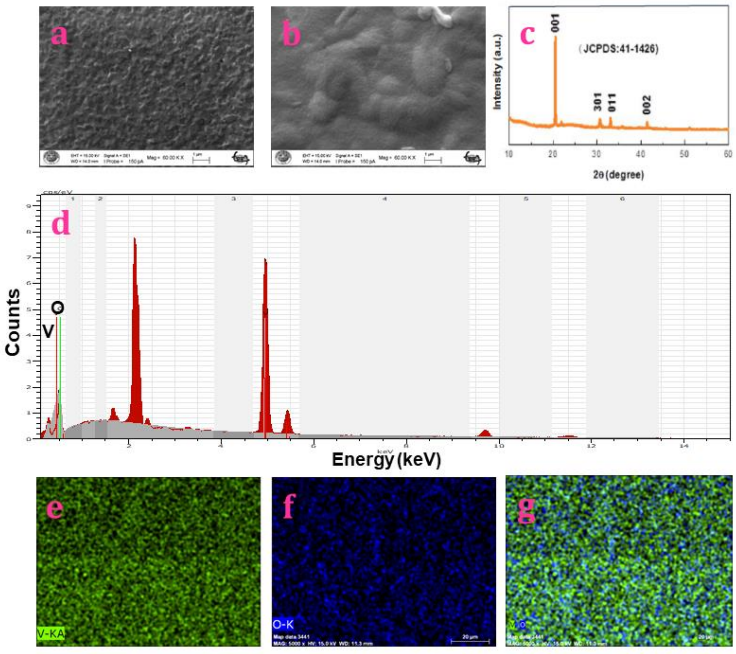


Figure 1. a) SEM image of bare SPGE b) SEM image of SPGE/V_2O_5 c) XRD pattern of V_2O_5 d) EDX spectrum of V_2O_5 e-g) Elemental mapping of V_2O_5 e) vanadium (green) f) oxygen (blue) g) overall mapping

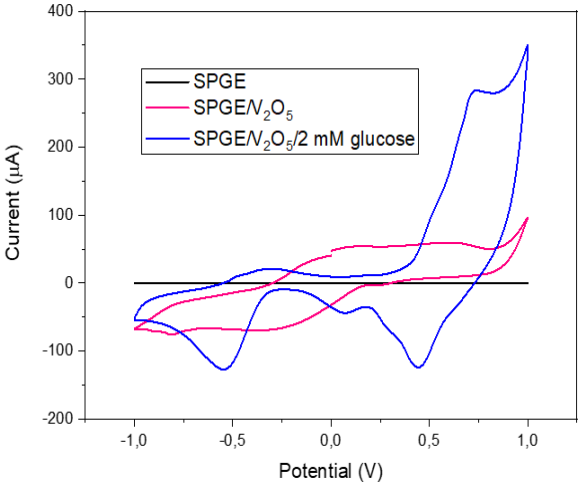


Figure 2. CV curves of SPGE and V_2O_5 modified SPGEs before and after addition of 2 mM glucose in 0.1 M NaOH solution at the scan rate of 100 mV s^{-1}

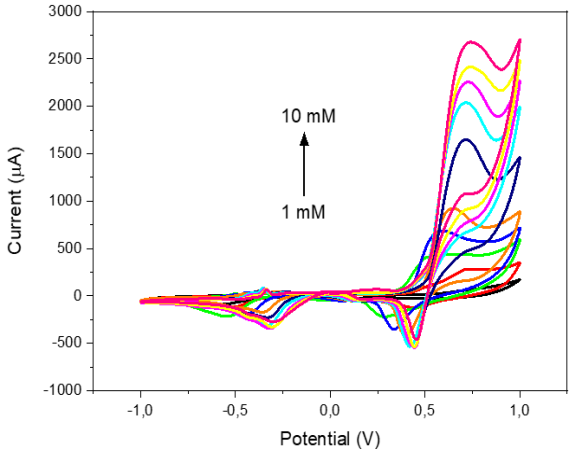


Figure 3. CV curves of V_2O_5 modified SPGE under various glucose concentration from 1 mM to 10 mM in 0.1 M NaOH solution at the scan rate of 25 mV s^{-1}

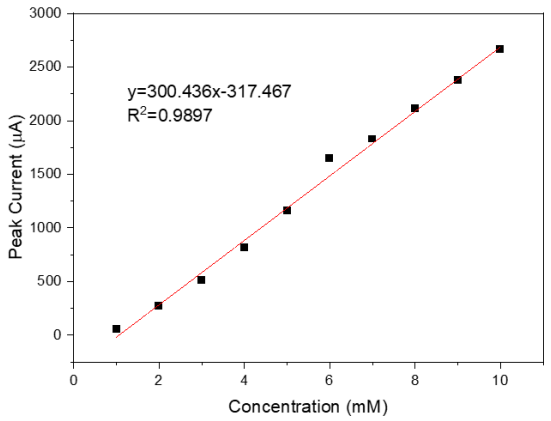


Figure 4. Calibration plot between the anodic peak currents vs glucose concentration

The observed phenomenon can be attributed to limitations arising from glucose absorption, potential intermediate presence and diffusion challenges within the diffusion layer. These factors collectively hinder the kinetics of the overall reaction, resulting in the positive shift observed in the anodic current. Furthermore, the accompanying figure demonstrates two cathodic peaks at +0.44 and -0.54 V following glucose addition, affirming both the outstanding electrocatalytic capabilities of the SPGE/V_2O_5 electrode and the irreversible nature of glucose oxidation (Fernandez et al., 2020).

The oxidation of sugars involves a dehydrogenation reaction, where the reactive group is typically a hemiacetal group found in all reducing sugars. It is proposed that the electrocatalysis process typically involves the attachment of the analyte onto the electrode surface, likely engaging d-electrons and vacant d-orbitals of the metallic substrate. Thus, it's highly likely that the adsorption of sugars onto the catalyst plays a pivotal role in initiating the oxidation reaction (Pérez-Fernández et al., 2016).

The SPGE/V_2O_5 electrode's sensing capability was monitored using CV. As illustrated in the Figure 3, each incremental addition of glucose correlated with an amplified CV signal. Within the 1–10 mM range, there existed a linear correlation between glucose concentration and the CV signal, demonstrating a detection limit of 0.9 µM. These performance metrics either match or occasionally surpass those previously reported in similar studies.

In summary, the process begins with the electrochemical uptake of glucose onto the SPGE/V_2O_5, followed by a dehydrogenation step. V_2O_5 film, formed at higher anodic potentials, significantly enhances the catalytic effect on glucose oxidation. The adsorbed glucose molecule can undergo direct oxidation to form gluconate, releasing OH^- along with the elimination of H^+. Alternatively, another pathway involves the conversion of dehydrogenated glucose into gamma-gluconolactone through oxidation, which further transforms into gluconate upon reacting with hydroxide ions. The electrode current's magnitude depends heavily on both the concentration of glucose and the pH of electrolyte, specifically the quantity of OH^- ions, as these ions are essential to counterbalance the protons produced in the dehydrogenation phase of the reaction (Grochowska et al., 2019). NaOH serves as a widely used electrolytic medium for non-enzymatic electrochemical sugar detection due to its crucial role demonstrated by OH^- ions in the reaction (Luo et al., 1996).

The oxidation peak current density observed during the reverse scan gradually rises with rising glucose content in the electrolyte. A graphical representation of the relationship between current (I) and concentration (c) was depicted in Fig.4. At lower glucose levels, a direct relationship between glucose concentration and current was established. Utilizing the equation: 3SD/slope (where SD denotes standard deviation and slope is obtained from the linear regression model) (Grochowska et al., 2019), a low detection limit of 0.9 µM was calculated.

Investigating interference effects is crucial in analytical science because it enables the distinction between interfering elements and a biomolecule with a similar physiological context within the biosensor. The sensor's selectivity was probed through CV using a consistent glucose concentration. The assessment focused on discerning the impact of various species that might disrupt sugar determination due to their close proximity in oxidation processes. Ascorbic acid, fructose and maltose were specifically studied as potential interfering species commonly found in food samples, usually at lower concentrations compared to sugars (Pérez-Fernández et al., 2016).

Figure 5a and 5b illustrate the electrode material's electrochemical response to various interfering species. When successively introduced into a 10 mM glucose solution, ascorbic acid, fructose and maltose showed notable differences in the current density of the oxidation peak recorded at +0.7 V.

As depicted in the Figures 5a and 5b, the interfering molecules undergo oxidation at the applied operational potential of +0.7 V. The current outputs resulting from the electrochemical oxidation of these interfering species might influence the analytical responses for glucose determination (Phetsang et al., 2019). Additionally, as observed in the Fig 5, the introduction of these mentioned interferences doesn't yield an increase in peak height at the glucose oxidation peak position in the CV profile, demonstrating the favorable selectivity of the resulting sensor (Hallaj et al., 2020).

In comparison to various non-enzymatic glucose sensors predominantly evaluated in alkaline environments, the performance of this glucose sensor matches or, in many instances, surpasses that of previously reported works, as detailed in Table 1.

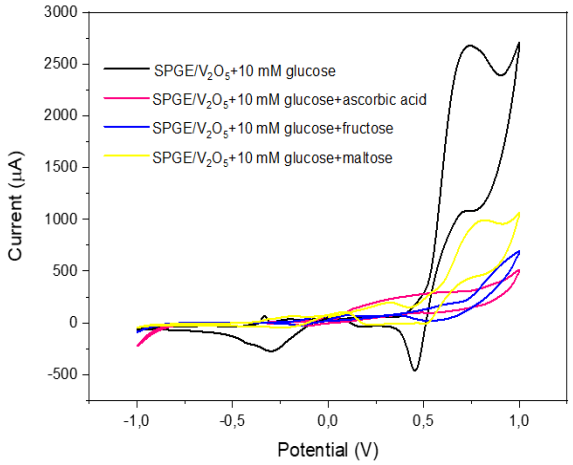


Figure 5a. The impact of biomolecules on interference

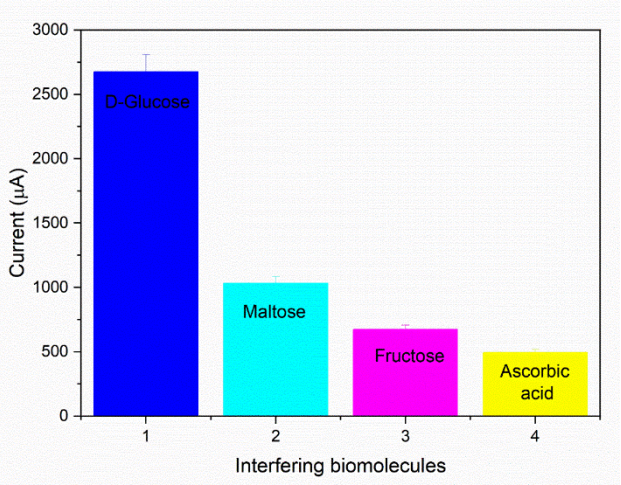


Figure 5b. Bar graph depicting the interference effect at +1.0 V

Table 1. Assessing the analytical performance of our proposed glucose sensor compared to various modified electrodes reported for glucose determination.

Catalyst	Morphology	Electrode	Chemical Environment	Linear range	Limit of detection	Selectivity	Ref
NiO	Hollow microsphere	GCE	0.1 M NaOH	0.005-0.364 mM	2 µM	AA, Urea, Leu, Pro, Lys, NaCl	(Cui et al., 2015)
Cu_2O	Nanoparticle	VC/graphite disk	0.1 M KOH	0-6 mM	2.4 µM	AA, KCl	(El Khatib and Hameed, 2011)
Co_3O_4	Nanofiber	GCE	0.1 M NaOH	0-2.04 mM	0.97 µM	AA, UA	(Ding et al., 2010)
Fe_2O_3	Nanoparticle	GCE	0.1 M PBS	0.0025-0.58 mM	0.58 µM	AA, UA	(Chen et al., 2014)
Mn_3O_4	Nanomesh	3D GF	0.1 M NaOH	0.1-8 mM	10 µM	AA, UA, AP	(Si et al., 2013)
$ZnO.V_2O_5$	Nanorod	GCE	100 mM PBS	1-1000 µM	125 250 µM	AA, Fruc, Dopamine, UA	Rahman et al., 2019
VO_x	Thin film	GCE	0.1 M KOH	1-10 mM	0.32 mM	AA, Acetominophen	Franceschini et al.,2023
V_2O_5@GO	Nanoparticle	Gold electrode	0.01 M NaOH	0.5-7.5 mM	0.859 mM	-	Prabakaran et al., 2024
V_2O_5	Thin film	SPGE	0.1 M NaOH	1-10 mM	0.9 µM	AA, Fruc, Malt	this work

Conclusion

A straightforward potentiostatic approach successfully enabled the preparation of a V_2O_5 thin film on the SPGE surface. This deposition technique facilitated the creation of well-dispersed V_2O_5 nanostructures on the electrode, significantly enhancing its electrocatalytic process in glucose oxidation during electrochemical assessments. These findings underscore the potential of V_2O_5 nanostructures as exceptional electrode materials for glucose sensing applications. The V_2O_5-modified SPGE exhibited an extensive linear range spanning 1 mM to 10 mM, boasting a detection limit as low as 0.9 μM for glucose. Notably, common interfering species found in physiological environments showed no discernible impact on glucose oxidation using SPGE/V_2O_5 electrodes. This suggests promising prospects for SPGE/V_2O_5 electrodes in non-enzymatic glucose sensing applications. Moreover, this study sheds light on the fascinating electrocatalytic behavior of V_2O_5 thin films and introduces electrodeposition as a safe and viable deposition method for V-based glucose electrocatalysts, marking a pioneering exploration in this field.

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Evaluation of Fruit Juices as Probiotic Delivery Systems: Challenges, Current Strategies and Health Benefits

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ABSTRACT

There is an increasing trend for development of alternatives to deliver probiotics with non-dairy products. Fruit juices have become one of main food products for delivery of probiotics. The availability of different fruit juice types, their fresh and healthy perception from the consumer's side and demand for plant-based products increase attention to fortification of fruit juices with probiotics. Yet, development of probiotic fruit juices is still an emerging area for the functional food concept. Probiotic juices can be developed by using both probiotic *Lactobacillus* and *Bifidobacterium* and their viability can be strain specific as well dependent on the utilized fruits. The transformation of the fruit components can play roles for the improvement of the potential health promoting functions of fruit juices which should be well-characterized. The insufficient viability of probiotic strains during shelf-life of fruit juices is one of the main challenges and efficient and relatively cheap encapsulation techniques should be developed to ensure their viability. In this study, recent achievements and developments to produce probiotic fruit juices have been summarized. Also, potential role of probiotic fortification for the health promoting functions of fruit juices related to probiotic metabolism has been discussed. Finally, strategies to increase the viability of distinct probiotics have been discussed.

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Introduction

In recent years, the functional beverage market is a rapidly growing area of the food industry due to the increasing interests of modern, health-conscious consumers for these products that can be effective to reduce disease risks and increase their quality of life (Corbo et al., 2014; Gupta et al., 2015). The demand for fermented plant-based beverages produced with different raw materials (fruits, vegetables, cereals etc.) is also increasing due to consumer's perception in the relationship between daily diet and healthy life as well as an increase in the number of individuals with a vegan preference. Fortification of the plant-based beverages with probiotics and conducting the fermentation processes with probiotic microorganisms especially with probiotic lactic acid bacteria (LAB) have become the method of choice for the development of functional plant-based products. These non-dairy raw materials are both cheaper and rich in phytochemicals, and they do not pose a health problem for individuals with lactose intolerance or cholesterol-restricted diets (Pereira and Rodrigues, 2018). In addition, vegetarian nutrition, and the desire to avoid allergic reactions caused by milk consumption have increased the demand for non-dairy probiotic products (Mojikon et al., 2022).

Lactiplantibacillus plantarum, Lactobacillus acidophilus, Lacticaseibacillus paracasei, and *L. brevis* are commonly used LAB species in fruit and vegetable fermentations. The probiotic functions of these species increase the attention to these strains in fruit and vegetable fermentations as they have positive effects on human metabolism with the consumption of these beverages (Guan et al., 2021). Probiotics are defined as "live microorganisms that, when administered in adequate quantities, confer a health benefit on the host by the United Nations Food and Agriculture Organization (FAO) and World Health Organization (WHO) experts in 2001. Several health benefits are associated with the probiotic consumption such as reduction in the intestinal pH, improvement of the intestinal microflora, helping to restore the natural microflora especially after antibiotic treatments, lowering cholesterol levels, reduction of ammonia and other toxic compounds, consumption of lactose, improvement of the digestion as well as the stimulation of

immune system. However, for probiotic microorganisms to show these benefits, at least 6 log CFU (colony forming unit) ml^{-1} of live microorganism must be taken at the time of consumption and probiotic strains should be able to maintain their viability, colonize and multiply in the host gastrointestinal tract (GIT). Many factors such as the food matrix, the properties of the probiotic strain, pH or storage conditions can have an impact on viability (Istrati et al., 2018; Andrade et al., 2019). If sufficient amounts of probiotic bacteria cannot reach the target area, the probiotic product is not expected to be beneficial (Aspri et al., 2020).

Fruits and vegetables are rich in vitamins (vitamin C and B-complex vitamins, provitamin A), minerals, aromatic compounds, carbohydrates, dietary fibers, phytochemicals, and polyphenols, as indicated in worldwide nutritional studies. The composition of these raw materials varies depending on the degree of ripening, the preparation stages of the fruits and vegetables for the process. While their water content varies between 70%-90%, they contain low amounts of oil in the pulp and shell parts, as well as in the core parts that are mostly not consumed. The protein content of fruits is variable but very low. Fruit juices help maintain blood pressure with their low potassium and sodium content. In addition, their low-fat content makes them suitable for consumption to maintain a healthy cardiovascular system (Rodríguez et al., 2021; Mojikon et al., 2022). As a result, the bioactive components of fruits have shown that they can reduce the risk of certain chronic and metabolic diseases when they are consumed regularly (Charlton et al., 2014).

It should be noted that there are also some problematic issues for fruits and vegetables that need to be solved to increase their consumption rates. Economic reasons (e.g., high cost-low affordability), changes in consumer behavior, scarcity of fresh fruit supply in some regions, short lifespan of fruits, seasonality and difficulty in storage are factors that affect fruit intake in public nutrition. These obstacles can be overcome by investments in research and development and the creation of appropriate regulations (Gerritsen et al., 2019; Rodríguez et al., 2021).

Fruits are sensitive to microbial spoilage due to their nutritional values and water activities and their shelf life is short. They are prone to spoilage by yeast and mold species, not by such bacteria, due to their low pH values. For this reason, fungi and LABs that can grow at high acidity values dominate the autochthonous microbiota of fruits. The safety of such products can be ensured by inhibiting spoilage species by pasteurization, cooking and chemical preservation methods. However, applied food processes can cause undesirable changes in the physicochemical and nutritional properties of the final product. One of the most used preservation methods is the addition of some chemical preservatives (potassium sorbate and sodium benzoate etc.). However, the presence of these preservatives does not meet the expectations of some consumers who want to consume food products with green labels (Plessas, 2021). To prevent these disadvantages, non-thermal food preservation methods (high hydrostatic pressure processing, pulsed electric field and ionizing radiation), smart packaging systems or natural antimicrobial preservatives can be used (Swain et al., 2014; Rodríguez et al., 2021). Development of plant-based fermented food products with LABs with GRAS status is a good alternative as a healthy preservation method accepted by consumers and has been used for centuries (Di Cagno et al., 2013). With the developing fermentation techniques, different microorganisms and substrates are used, and various end products are formed (Melini et al., 2019). These fermentation techniques enable the production of functional beverages with various properties by fermenting fruits and vegetables in recent years. By fermentation, various desirable aromatic compounds such as amino acids, organic acids can be produced, while sources of undesirable flavor such as olefinic in vegetables can be reduced, nutritional values as well as organoleptic quality of the products can be improved (Guan et al., 2021). In addition, metabolites such as organic acids, carbon dioxide, ethanol, hydrogen peroxide, bacteriocins and some fatty acids produced help to provide biological protection (Di Cagno et al., 2013). In a study, antioxidant, carotenoids, and phenolic contents of fermented orange juice by different strains of *L. brevis* and *L. plantarum* were determined. Compared to the unfermented sample, it was observed that the fermented beverage had higher antioxidant capacity, while it was observed that different microorganisms used had different effects on the amount of carotenoid and phenolic compounds formed (de la Fuente et al., 2021). In a study conducted by Yang et al. (2018), the antioxidant capacity and physicochemical properties of apple, pear, carrot mixture fermented by two *L. plantarum* strains were tested and it was concluded that the antioxidant capacity in the fermented product was high, and the product was suitable for consumption.

Microorganisms alter the food matrix and produce various bioactive components during the fermentation of fruits and vegetables which results in the development of the nutritional and sensory properties of the final product (Garcia et al., 2020). In addition to the pleasant taste and smell of fruit and vegetable drinks, organic acids, phenolics and vitamins produced as a result of fermentation also increase their preference in terms of consumption (Ghosh et al., 2015). Some fruit juices with probiotic properties produced with fermented systems are already on the market (Table 1). Fruit juices are important because of their fresh food appearance and being suitable carrier environments where probiotics can show beneficial effects on health. In this system, it is promising to reach a wide consumer mass with a well-designed product (Aspri et al., 2020). With this study, it was aimed to reveal the important points in the production of fermented fruit juices by explaining the relationship between the environment provided by the fruit matrix and the selected microorganisms. In this context, mostly studied fruits and microbial species were brought together and methods that would ensure the continuity of the viability of these species in the final product were reported.

Lactic Acid Metabolism and Its Importance in Fermented Beverage Production

Fruit juices are often preferred by people of all age groups with their nice refreshing tastes. Fruits are rich food sources in terms of important nutritional components (antioxidants, minerals, and vitamins), and due to the natural sugars in their structure, they can allow probiotic LAB to thrive in these environments. Probiotic

microorganisms grow by using carbon sources present in plant-based raw materials such as glucose, fructose, galactose, sucrose, maltose, mannitol and raffinose for their metabolic activities. Fruit juices may be easier to digest in the stomach environment than dairy products. Thus, less time spent in the stomach environment with high acidity, especially in fermented products where a probiotic effect is expected, can increase the number of live microorganisms that can reach the digestive tract (Pereira and Rodrigues, 2018; Mojikon et al., 2022). Fermentation of fruits and vegetables is a widely preferred method, but they are less favorable environments for microbial activity than meat and dairy products. These environments are less favorable especially due to their acidity levels, indigestible components, and some bioactive components (Di Cagno et al., 2013; Balthazar et al., 2018; Li et al., 2021a).

Table 1. Fermented fruit juices in commercial production

Kind of fruit	Probiotic microorganisms	Commercial origin and name
Orange-red grapefruit Orange-mango Strawberry-lime-mint Pear-apple-matcha Pineapple-apple-ginger	*L. plantarum* 299v	ProViva-Sweden
Blueberry acai Pomegranate- blackberry Mango Strawberry-banana Raspberry-blackberry Mango-orange Orange	*L. plantarum* 299v	GoodBelly Probiotics-USA
Orange-mango Apple-pear	*L. rhamnosus* GG	Biola-Norway
Sparkling lemon-apple juice Blackberry-apple-lemon Peach-apple-lemon Apple cider vinegar-red beet Apple cider vinegar-turmeric-ginger Apple cider vinegar-kale lemon Apple cider vinegar-cinnamon Apple cider vinegar-chili-ginger-lime	Water Kefir Culture *Bacillus coagulans* Gbi-30 6086	Kevika-USA
Apple-ginger Pineapple-carrot Orange-peach Apple-grape Orange-grape-mango-peach-passion fruit Orange-grape-sea buckthorn-carrot Blueberry-raspberry Pineapple-coconut Orange-mango Mango-apple-orange-banana-passion fruit	*L. rhamnosus* GG	Valio Geofilus-Finland
Apple-mango	*L. paracasei* 8700:2 *L. plantarum* HEAL 9	Healty life-Australia
Grape-orange	*L. paracasei*	Malee probiotic juices, Thailand
Apple-mango-pineapple-banana Mango lemonade Peach-passion fruit	*Bifidobacterium lactis* HN019	Tropicana - USA
Lemon-strawberry-dragon fruit	*B. coagulans*	Press- UK
Mango-passion fruit Strawberry-watermelon	*L. casei* *Bifidobacterium*	PERKii probiotics-Australia
Strawberry-raspberry-lemon-tart cherry juices Coconut-pineapple-ginger-turmeric-orange-lime Juices Pineapple-cucumber-spinach-celery-ginger-kale-collard green-lemon juices	*B. coagulans* Gbi-30 6086	Suja Organic-USA

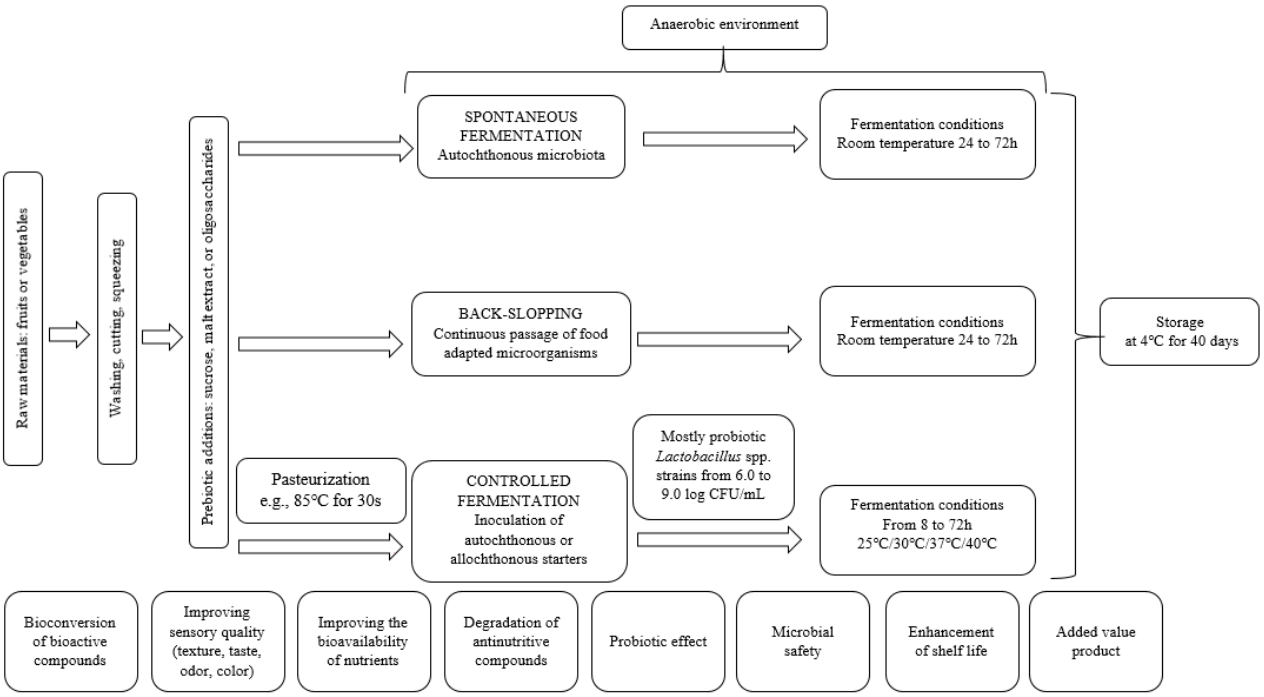

Figure 1. Fermented fruit juice production flow chart (Szutowska, 2020)

Fruit fermentation can occur spontaneously with autochthonous LAB under suitable environmental conditions. As spontaneous species, *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Weissella* spp., *Fructobacillus* spp., and *Enterococcus* spp. are common. Another method in which the fermentation of the raw material is carried out by inoculating it with a part of the previously fermented product is the back-slopping method. This method prevents unsuccessful fermentation in the face of changing microbiota and improves the quality of the product. The continuous use of the fermented substrate allows the selection of the strain that is best adapted to the environment and the implementation of a starter culture-like application. Although these two methods are preferred in the production of traditional fermented products, it is more reliable to use defined starter cultures with potential probiotic functions (Ilango and Antony, 2021). For this reason, controlled fermentation is preferred for a reliable, reproducible, and standard quality product. The preferred starter cultures in this process are LABs, e.g., *L. plantarum*, *L. rhamnosus, L. gasseri* and *L. acidophilus* (Rodríguez et al., 2021). The most important criterion for effective fermentation is the microbiota of the fruit. It is necessary to investigate suitable microorganisms that can be effective in the food matrix. In particular, the fermentative properties of microbial strains isolated from the fruit's own microbiota responsible for spontaneous fermentation are higher (Li et al., 2021a).

The only difference from the traditional method in obtaining a probiotic juice is the inoculation of the probiotic culture. The juice processing process begins with the cleaning, sterilization, and classification of the raw material. Afterwards, the fruit flesh is broken down and the juice yield is increased. Depending on the raw material, the juice mass can be cloudy, viscous, and dark in color, containing colloidal components due to polysaccharide compounds such as pectin or starch. With the clarification process, this situation is adjusted according to the desired quality criteria. This process includes removal of solid particles by filtration, depectinization with pectinolytic enzymes or degradation of starch with amylase. The solid particles formed after the process are removed by conventional methods or ultrafiltration. Finally, gelatin, bentonite thinners are used and diatomaceous earth or kieselguhr filters are used to make the clarification process more active. Membrane processes such as microfiltration or ultrafiltration have been preferred recently (Bhattacharjee et al., 2017; Urošević et al., 2017). After this stage, heat treatment is applied to the raw fruit juice. Although it is classified according to the applied heat treatment temperature, pasteurization (<100 °C) is mostly preferred for fruit juices. By heat treatment, inactivation of the flora from the microbiota of the fruit and unwanted enzymes (polyphenol oxidase, peroxidase, pectin esterase, polygalacturonate) can be achieved. To ensure a controlled fermentation in fermented juice production, the main mass temperature is cooled to the appropriate temperature for the development of the selected culture. At the end of the incubation period, fruit juices are stored at cooling temperature (4–10°C) (Petruzzi et al., 2017; Pimentel et al., 2019). Figure 1 demonstrates the production of the fermented juices with the methods explained above and the potential technological and functional roles of the probiotic inoculation for the juice production (Szutowska, 2020).

Lactic acid fermentation is used as a preferred method to preserve the safety, nutritional and sensory properties of fruits and vegetables. It is known that LAB increases the flavor of fermented products with various compounds which form as a result of their metabolism (Fan and Hansen, 2012; Zheng et al., 2020). Homofermentative LAB produces lactic acid as the main end product of carbohydrate fermentation, while heterofermentative LAB produces various products such as acetic acid, carbon dioxide, ethanol, acetoin, and diacetyl (Leroy and De Vuyst, 2004; Gänzle, 2015). Various volatile compounds are produced as a result of fermentation utilized by LABs that can provide specific flavors and aromas to the final product.

Microbial strain, juice composition (acidity, carbohydrate content, nitrogen sources, mineral substances) and possible interactions between microorganisms and food components can be counted as the challenging factors affecting the viability of probiotics. In this case, we can categorize various factors affecting the viability of probiotics under four headings. First, the factors in the environment that occur in relation to the fruit itself; fruit, pH, titratable acidity, molecular oxygen, water activity, presence of components such as salt and sugar, artificial flavoring and coloring agents. The second group includes microbiological parameters; characteristics and inoculation rate of selected probiotic strains. Finally, environmental factors related to food processing parameters; heat treatment, incubation temperature, cooling rate, selection of packaging material, storage methods and conditions and finally presence of oxygen (Tripathi and Giri, 2014; Perricone et al., 2015), and the last one is sensory properties (Perricone et al., 2015, Lebaka et al., 2018).

Challenging factors affecting the viability of probiotics

Properties of the raw material

The first effective factor in ensuring probiotic vitality is the selection of fruit itself. Fruits and vegetables are naturally rich in water-soluble vitamins, minerals, carbohydrates, and dietary fiber. In addition to these nutritional components, they are also valuable in nutraceutical terms because they contain phytochemicals and polyphenols such as anthocyanins or carotenoids (Shah and Singhal, 2017; Istrati et al., 2018). The chemical composition of fruits can directly affect the viability of the probiotic culture. While protein and dietary fibers in the structure can protect bacterial cells from acidic stress, citric and malic acid might affect its adversary. The presence of phenolic compounds affects probiotic viability. It has been reported that the presence of high concentrations of phenolic compounds may cause loss of viability (Perricone et al., 2015). Benzoic acid, an important phenolic acid found in fruits, is used as a preservative in many foods. Previously it was suggested that, due to the very low pH and high benzoic acid content of cranberry juice, probiotic viability might decrease during shelf life (Shori, 2016; Pimentel et al., 2019). On the contrary, in a study in which passion fruit was used in the production of probiotic fruit juice and green tea was added to this fruit juice, the increase in the amount of bioactive compounds positively affected the growth of *L. gasseri* (Lima et al., 2022).

High levels of acids such as malic and citric acids (11 mg/L) and fibers (2.8 g/L) in the structure of orange juice are important in terms of being metabolized by some cultures, so orange juice can provide a suitable environment for the continuation of probiotic vitality. In a study conducted with orange juices in which *L. plantarum, L. rhamnosus, L. paracasei* and *L. brevis* species were used as probiotic strains, all strains were able to maintain their vitality during storage by converting malic acid to lactic acid by malolactic fermentation (Multari et al., 2020). Other LAB strains such as *Pediococcus acidilactici* were also shown to survive in fruit juices as reported for strain CE51 in concentrated orange juice with a number of survivals around 8.5 log CFU mL^{-1} at the end of the storage period (de Oliveira et al., 2021). In another study, *L. rhamnosus* was used as a probiotic strain in orange juice and 6 log CFU mL^{-1} microbial count was obtained after 28 days of storage at 4 °C (Sengun et al., 2020). As a result of fermentation of pomegranate juice with *L. plantarum* ATCC 14917, it was reported that the viability reached as high as 10 log CFU mL^{-1} in the first three weeks and this value was 8.83 log CFU mL^{-1} in the last week of storage. This situation has been associated with lactic acid fermentation with increased bioaccessibility of phenolic compounds that can act as prebiotics (Mantzourani et al., 2019).

The pH value is one of the main factors affecting the microbial viability and sustainability of fermented fruit juices. Fruits naturally contain high levels of organic acids in their structure. The low pH value of the medium increases the concentration of their undissociated forms. Consequently, it is hypothesized that the combined effect of acidic conditions and the intrinsic antimicrobial activity of acids affect probiotic viability. Among LABs, *Lactobacillus* are generally more resistant and can survive at pH values of 3.7-4.3, while *Bifidobacterium*, another important group, are less tolerant and even around pH 4.6 may be unfavorable in some cases (Tripathi and Giri, 2014; Perricone et al. 2015; Patel, 2017). The chemical composition of the fruit and its juice is important in maintaining microbial viability. In particular, protein and dietary fiber in the structure have a protective effect on bacteria against acidic stress. Protein, which causes turbidity in the production of clear fruit juice, is removed from the environment by using components such as gelatin and bentonite. For this reason, especially in clear fruit juices, microbial vitality can be lost to a great extent due to protein deficiency. It has been reported that the minimum protein concentration should be 0.3% for the preservation of viability (Nualkaekul and Charalampopoulos , 2011; Pimentel et al., 2019).

Probiotic strains in the fermented system

Fruit matrices are suitable environments for the growth of probiotic bacteria, but for microorganisms to maintain their viability, they must first protect themselves from the natural acidity conditions of the fruit, and also need to adhere to the fruit matrix and compete with pathogenic and spoilage microorganisms (Di Cagno et al., 2013; Rodríguez et al., 2021). Possible reasons that prevent the growth of probiotic LAB in these environments: first, high acidity, presence of oxygen in the system, lack of free amino acids, short chain peptides and oligosaccharides can be counted. For these reasons, the selection of strains that can maintain their stability, vitality and functionality in the system is important for fruits and vegetables, which have more challenging environments than other common fermented products (Žuntar et al., 2020). Microbial flora is an important factor in the development of fermentation and quality in the product, therefore, it is necessary to investigate suitable microorganisms for the biological transformation of the target food matrix, which is the environment where fermentation will take place (Pereira and Rodrigues, 2018).

The carbon sources used by LAB in metabolic activity differ between strains. In general, this metabolism may depend on the composition of the food and the

fermentation conditions. Fruits are a good source of fermentable sugars (glucose, fructose, galactose, and sucrose etc.). The main energy source for many bacteria is glucose, however, fructophilic lactic acid bacteria (FLAB), a subgroup of lactic acid bacteria, use fructose as their main energy source. These bacteria, which belong to the genus *Fructobacillus* spp, have poor glucose metabolizing abilities, but grow well in fructose-rich environments (Garcia et al., 2020; Mojikon et al., 2022). In a study where *L. casei* was used in a local fruit fermentation unique to Brazil and the fermentation conditions were examined, it was reported that fructose was the most metabolized sugar (Pereira et al., 2017).

Two methods that can be preferred to carry out a controlled lactic acid fermentation in vegetables or fruits; it is the use of autochthonous starters isolated from the same raw material matrix, or the use of allochthonous bacteria isolated from a particular raw material and used to ferment various products (Di Cagno et al., 2013). For the fermentation of fruits and vegetables, non-autochthonous mostly animal-derived distant species are used, and the viability of these microorganisms decreases during the storage process. For the allochthone/commercial starter cultures, high performance strains are needed to guarantee fermentation on an industrial scale as they have limitations such as not considering other properties other than accelerating acidification, poor adaptation to the main sensory and functional properties of the food matrix, being metabolically inflexible and not reflecting the ecosystem in which they are found. Microorganisms to be isolated from environments obtained by spontaneous fermentation of fruits and vegetables are important as they can guarantee a longer shelf life as autochthonous cultures and have adaptive advantages that can provide nutritional, sensorial, and rheological enhanced properties (Di Cagno et al., 2013, Rodríguez et al., 2021). Table 2 shows the preferred fruits and bacterial strains in fermented juices. To produce a probiotic fruit juice, either an acid-tolerant microbial strain is added to the fruit juice, or it is fermented with probiotic microorganisms.

The fermentation process is advantageous over the addition of microbial strains because, depending on the development of the added strain during fermentation, a product with a lower sugar content and the development of an adapted strain that can maintain its viability are provided. In addition, metabolites such as bacteriocins synthesized by selected species improve product quality during storage (Pereira and Rodrigues, 2018).

The method and the level of addition of probiotic cultures affect their survival. Generally, the preferred method starts with the activation of lyophilized probiotic cultures in appropriate media. Afterwards, the biomass obtained by centrifugation is washed three times in sterile saline solution and dissolved in this solution again and inoculated into the fermented system at a certain rate (~2%). Direct addition of cultures with currently used aseptic dosing technologies does not require the propagation process, and it provides an advantage in terms of time, while the use of high concentrations to reach the minimum viability creates a cost disadvantage. In addition, it is important to ensure the stability of the environment for minimum viability. Addition of a high concentration of culture may also cause physicochemical and sensory changes in proportion to the amount used (Istari et al., 2018; Pimentel et al., 2019).

Environmental factors affecting the survival of probiotics in the fermented system

The viability of probiotic microorganisms in fruit and vegetable juices depends on many factors such as oxygen level, pH, antimicrobial components, strain type, substances in the nutrient medium and temperature. However, survival of probiotics in fermented fruit and vegetable juices is more difficult during storage compared to fermented milk products due to the food matrix (Lillo-Pérez et al., 2021).

Probiotic viability and potency of fermented fruit juices are affected by various factors. Some of the suggested methods for incorporating probiotic cultures into the food matrix to ensure their continuity until consumption and passage to the gastrointestinal tract are fortification with prebiotics, adaptation and induction of resistance, storage under refrigeration and use of antioxidants as well as microencapsulation (Lillo-Pérez et al., 2021). One of the most effective ways to ensure probiotic stability in fruit juice is to enrich the environment with prebiotics or protective components (Perricone et al., 2015). Prebiotics are defined as non-digestible components that are selectively used by certain bacteria in the colon to support their growth and improve host health by providing health benefits. The two most preferred approaches for providing prebiotic activity in fruit juices are addition of the prebiotic carbohydrate directly to the food matrix and synthesis of the prebiotic carbohydrate in fruit juice. The direct addition of purified oligosaccharides as prebiotics can increase production costs. If it is synthesized directly in the environment, it suppresses the purification steps and uses the natural sugars of the fruit juice. Converting simple sugars to prebiotics can reduce the sugar content of the product. However, it is of great importance that the prebiotic compounds should be stable in the food matrix and during the applied processes (temperature, low pH etc.). In addition, if prebiotics are reducing sugars, they can reduce the prebiotic activity of carbohydrates by supporting Maillard reactions (Charalampopoulos & Rastal, 2012; Fonteles & Rodrigues, 2018). Khezri et al. (2018) reported that addition of inulin to the fig juice resulted in an increment of the numbers of *L. delbrueckii* as well as increased antioxidant capacity and organoleptic properties for the inulin added juice sample was observed. Saarela et al. (2006) stated that oat flour and β-glucan added to apple juice preserved probiotic species during storage.

It has been determined that exposing probiotic bacteria to a non-lethal stress will enable them to gain some kind of resistance and that their response to stress can be induced (Gobbetti et al., 2010; Perricone et al., 2015). For instance, Perricone et al. (2014) aimed to reduce the vitality loss of *L. reuteri* DSM 20016 in different fruit juices against low pH and phenolic compounds according to this approach. For this purpose, strains were cultivated by preparing media with different properties (pH and phenolic acid variables) in the laboratory environment. As a result, they showed that they were able to extend the viability of *L. reuteri* DSM 20016 by 5 days against phenol stress and 11 days against acid stress.

Table 2. Microorganism species and fruits used in fermented systems

Fruit Juice	Microorganism	Reference
Blueberry juice	*L. plantarum*	Zhang et al., 2021
Citrus juice	*L. plantarum* SI-1; *L. pentosus* MU-1	Yuasa et al., 2021
Pineapple juice	*B. lactis* Bb12*; L. plantarum* 299V; *L. acidophilus* La5	Nguyen et al., 2019
Kuntze Fruit *(Elaeagnus angustifolia var. orientalis* (L.)	*B. animalis* subsp. *lactis* HN-3	Wang et al., 2022a
Blueberry pomace juice	*L. rhamnosus* GG; *L. plantarum*-1; *L. plantarum*-2	Yan et al., 2019
Broccoli juices	*Pediococcus pentosaceus*	Xu et al., 2021
Jujube juice	*L. plantarum* CICC20265; *Bifidobacterium breve* CICC6184; *Streptococcus thermophilus* CICC6220	Xu et al., 2019
Blueberry and blackberry juices	*L. plantarum* BNCC 337796 *Streptococcus thermophilus* CGMCC 1.8748; *Bifidobacterium bifidum* CGMCC 1.5090	Wu et al., 2021
Kiwifruit juice	*L. acidophilus* 85 (La85)*; L. helveticus* 76 (Lh76) *L. plantarum* 90 (Lp90)	Wang et al., 2022b
Ginkgo kernel juice	*L. acidophilus* BNCC 185342*; L. plantarum* BNCC 337796*; L. casei* ATCC 393	Wang et al., 2019
Carrot juice	*L. plantarum* NUC116	Wan et al., 2019
Grape juice	*O. oeni* MS9 *and* MS46	Del Valle et al., 2022
Sohiong juice	*L. plantarum* MCC 2974	Vivek et al., 2019
Carrot-orange juice	*L. acidophilus* CECT 903 (ATCC4356)	Valero-Cases & Frutos, 2017
Blueberry juices	*L. plantarum; L. fermentum* *L. plantarum* BNCC337796	Li et al., 2021a
Melon Juice	*L. delbrueckii* subsp. *Bulgaricus; L. paracasei* subsp. *paracasei* 34; *L. rhamnosus; L. lactis* subsp. *cremoris* 660; *L. lactis* subsp. Lactis*; S. salivarius* subsp. *thermophilus*	Rúa et al., 2018
Apple juice	*L. plantarum* NCIMB 8826	Roberts et al., 2018
	L. paracasei subsp. *paracasei*	Pimentel et al., 2015
	L. acidophilus TISTR 1338; *L. casei* TISTR 390 *L. plantarum* TISTR 543	Kaprasob et al., 2017
Mediterranean fruit; Juices (Apple- quince- grape- kiwifruit- prickly pear-pomegranate)	*L. fermentum; L. kefiran; L. lactis; L. mesenteroides; Saccharomyces cerevisiae*	Randazzo et al., 2016
Pineapple (*Ananas comosus* L. Merril) and Jussara Fruit	*L. rhamnosus* GG	de Andrade Pires et al., 2020
Cupuassu Fruit *(Theobroma grandiflorum)*	*L. casei* NRRL B-442	Pereira et al., 2017
Apple Juice	*L. acidophilus; L. plantarum; L. fermentum*	Peng et al., 2021
Prickly pears	*L. fermentum* ATCC 9338	Panda et al., 2017
Pomegranate (Punica granatum)	*L. casei* subsp. *casei (*NRRL B-1922*)*	Mustafa et al., 2020
Sweet Orange (Citrus sinensis) juices	*L. plantarum* B42; *L. rhamnosus* B68 *L. paracasei* B37; *L. brevis* DSM32386	Multari et al., 2020
Sweet melon (Cantaloupe)	*L. plantarum* FBS05	Muhialdin et al., 2021a
Watermelon	*L. plantarum* DSM 9843 *(Lp299v®)*	Kanafusa et al., 2021
Jackfruit Juice	*L. casei* ATCC334	Muhialdin et al.,2021b
Dragon fruit juice	*L. plantarum* FBS05	Muhialdin et al., 2020
Mango juice	*L. rhamnosus* GG	Moreira et al., 2017
Orange juice	*L. casei*	Miranda et al., 2019
Black Chokeberry and Sea buckthorn juices	*L. plantarum (*DSM 16365, DSM 20174, DSM 10492, DSM 100813), *O. oeni* strains LAB6, LAA1 and B2013	Markkinen et al., 2019
Cornelian cherry	*L. paracasei* K5	Mantzourani et al., 2019
Goji berry juice	*L. plantarum* strains (LP 39, Lp goji and C8-1*)* *L. acidophilus* strains (NCFM, 6081 6075) *L. helveticus* strains (6024 and LH, LH10)	Liu et al., 2022a
Sea buckthorn juice	*L. acidophilus* La-26 *L. delbrueckii subsp. bulgaricus* Lb-57 *L. casei* Lc-630; *L. plantarum* Lp-6	Liu et al., 2022b
Jujube juice	*L. acidophilus* 85; *L. casei* 37; *L. helveticus* 76; *L. plantarum* 90	Li et al., 2021b
Mulberry juice	*L. plantarum* Lp-115™ (ATCC SD5209) *L. acidophilus* La-14™ (ATCC SD5212) *L. paracasei* Lpc-37™ (ATCC SD5275)	Kwaw et al., 2018
Coconut water	*L. plantarum* DW12 *L. brevis* CRL 2050; *L. brevis* CRL 2051	Kantachote et al., 2017
Cherimoya Juice	*L. plantarum* CRL 2030; *L. rhamnosus* CRL 2049; *F. tropaeoli* CRL 2039	Isas et al., 2020
Peach juice	*L. acidophilus* PTCC 1643; *L. fermentum* PTCC 1744	Hashemi et al., 2021

LABs are very sensitive to fluctuations in storage temperatures. While high temperature values change the microbial activities and growth of probiotics, low temperatures may reduce their metabolic activities and cause inhibition of cellular development (Lillo-Pérez et al., 2021). In this process, cooling may provide longer survival, while thermal stress may cause detrimental effects and cause probiotic species to lose their viability (Patel, 2017).

Probiotic strains preferred in fermented systems are generally anaerobic or microaerophilic in terms of oxygen needs. For this reason, changes in the oxygen level during storage may affect their viability and functionality. In addition, the presence of reactive oxygen species (ROS) such as oxygen, H_2O_2 or superoxide ions can cause oxidative damage. *Bifidobacterium* are often more sensitive to such conditions than LAB. In this context, the effect of food packaging is important, and although glass packages are more advantageous due to their low oxygen permeability levels, they can be used in a limited way due to their high cost and fragility. When packaging materials other than glass material are preferred, it is recommended to use probiotic strains that are more resistant to oxygen, to include antioxidants in the packaging material, to change the atmosphere in the product by increasing the CO_2 content of the headspace in the packaging, and to develop multi-layer packaging designs with selective permeability (Patel, 2017; Pimentel et al., 2019).

Sensory Properties

Probiotics fruit juices have limitations in terms of their overall acceptability and sensory attributes. Additionally, sensory evaluation is also commercially important. Therefore, when developing probiotic juice, it is important to consider acceptability in terms of appearance, flavour, texture and taste (Naseem et al., 2023). The sensory qualities of probiotic foods that are non-dairy can be influenced by interactions between various probiotic strains and food substrates, where textures, flavors, aromas, and colors can be enhanced or aggravated by the production of different metabolic compounds, such as lactic acid and other metabolites, during processing and storage (Gomes et al., 2021). Some studies have shown that the addition of probiotics does not negatively affect the sensory acceptability of fruit juice (de Souza Neves Ellendersen et al., 2012; Ryan et al., 2020; Kardooni et al., 2023). The results of a descriptive sensory analysis conducted in a study of orange juice containing probiotics and prebiotics showed that the functional juices were described as “dairy” aromas, and “dirty,” “medicinal,” “artificial,” and “earthy” flavors, distinguishing them from the conventional juices (Luckow & Delahunty, 2004). In another study conducted, mango juice produced using different probiotic strains. While the sensory results of mango juices produced with some of these strains were positive, mango juices produced separately with *L. plantarum* and *L. rhamnosus* were described as ‘bitter’, ‘sour’, ‘aftertaste’, and ‘off-flavor (Mandha et al., 2022). In a study with passion fruit juice, fermented fruit juice was mainly correlated with the terminologies “salty, acidic and bitter tastes” and “sweetener aftertaste” (Fonseca et al., 2022). Reducing undesirable taste and odor in non-dairy probiotic products can be masked by the addition of desired taste or volatile molecules. The addition of tropical fruit juices such as pineapple, mango and passion fruit can significantly affect the taste and aroma of the final product (Luckow et al., 2006; Naseem et al., 2023). In addition, Aziz et al. (2023) added 8% sucrose to fermented pineapple juice in order to reduce the sour taste and this increases the overall acceptability of juice (Aziz et al., 2023).

Current strategies to ensure the probiotic viability

Although the viability of probiotics is affected by many other factors, the strain used is important and the most suitable strain that can adapt to environmental conditions by meeting the requirements should be selected. Environmental factors such as high acidity and low temperature can also affect the metabolism and growth of the probiotic in the matrix. The presence of certain substances in probiotic fruit juice or the external addition of these substances can contribute to vitality. Fermentable sugars, phenolic compounds, antioxidants, proteins, and prebiotics found naturally in fruit juice or added later can contribute to maintaining vitality. These substances can protect probiotics from damage caused by stomach acidity (Patel, 2017; Mojikon et al., 2022; Plessas, 2021). In a study in which passion fruit was used in the production of probiotic fruit juice and green tea was added to the fruit juice, the amount of phytochemicals in the environment increased, and as a result of this increase, the development and viability of *L. gasseri* was supported (Lima et al., 2022). The addition of prebiotics also increases probiotic activity and viability. Ascorbic acid, on the other hand, can contribute to vitality by providing the environment desired by probiotic bacteria by showing antioxidant properties and preventing oxidation (Tang et al., 2023).

Andrade et al. (2019) conducted a study in which they evaluated the survival rate of a probiotic strain, *L. rhamnosus* ATCC 7469, in guava juices with simulated gastrointestinal conditions during refrigerated storage. In this study, they reported that the initial survival rates of bacteria were higher in unfermented media in fruit juices fermented by adding inulin and stevia, but there was a lower decrease in viability and survival in fermented juices after 28 days of storage. This showed that fermentation can improve stability in storage. In another study, in which *L. rhamnosus* was preferred, fermented and unfermented passion fruit was used, and they reported higher bacterial survival rate in fermented juice in simulated gastrointestinal environment (Farias et al., 2016). Nguyen et al. (2019) modelled the survival of probiotics in fermented pineapple juice and found that the probiotics showed 10 times higher survival against stress factors, such as after treatment with 0.3% pepsin and 0.6% bile salt in comparison to the unfermented sample. The increase in bacterial survival is associated with the final pH after fermentation. Bacteria that can survive at low pH have increased their ability to survive in the gastrointestinal environment, as they encounter pH pre-adaptation (Mathipa & Thantsha, 2015). The fermentation process can eliminate potential anti-nutritional factors present in foods by producing flavors, aromas, and desired textures, as well as increasing bacterial survival time (Swain et al., 2014).

Various strategies have been developed to increase and maintain probiotic viability. These include emulsification,

encapsulation, ultrasound, pre-adaptation applications, use of high acid fruit juice, use of more than one fruit juice. The use of high-acid fruits in the production of probiotic juice may reduce the negative effects that the probiotic bacteria may encounter in the host by providing a pre-stress. In probiotic fruit juices produced using more than one fruit juice, a fruit juice with a high pH value is added to the juice with a low pH value in order to increase the viability of the probiotic. Thus, by obtaining a relatively higher pH value, the viability of the probiotic strain is increased. In this respect, carrot juice is a fruit juice to be preferred due to its high pH value (Plessas, 2021).

Encapsulation is an application that protects bioactive components from all kinds of harmful factors in the environment. The use of microencapsulation technology to protect probiotic species from the damage they are exposed to in the external environment has provided successful results. Studies have shown that this method is more suitable for providing an anaerobic environment, while creating a physical barrier against harsh environmental conditions (temperature, pH, gastric acid, bile salt etc.), especially for sensitive probiotics (Perricone et al., 2015). By using various encapsulation agents (alginate, κ-carrageenan, resistant starch, inulin etc), vitality is maintained both in the food matrix and in the digestive tract until it reaches the host gut. Thus, the shelf life of the probiotic juice produced can be increased. For this purpose, methods such as spray drying, lyophilization and emulsification are used. Spray drying is preferred especially in probiotic fruit juice production due to its low cost, high capacity, and efficiency method (Plessas, 2021; Tang et al., 2023; Vivek et al., 2023).

In a mixed fruit juice enriched with probiotic *L. rhamnosus* LPAA 01, *L. casei* LPAA 02 and *L. plantarum* LPAA 03 strains microencapsulated by spray drying using maltodextrin, microcapsules were evaluated for their physicochemical properties and microbial viability and stability. As a result, it was reported that the number of viable cells were > 6 log CFU g^{-1} up to 20 days at 5 °C, and the physicochemical properties were within acceptable limits for 14 days at 25 °C (Souza et al., 2021). da Silva et al. (2021) produced microcapsules containing probiotic *L. acidophilus* LA-02 with a complex preservation and then cross-linking method with transglutaminase, and investigated the effects and viability of these added fruit juices on fruit juice during storage. They reported that after 63 days of storage at 4 °C, orange juice was the most suitable medium and the protective effect of microcapsules on the probiotic strain was promising. In a study using a different approach, Mantzourani et al. (2018) added probiotic *L. plantarum* to Cornelian cherry fruit juice as wheat bran immobilized cells and in free form. As a result, they found that the viability (9.95 log cfu mL^{-1} at the 4^{th} week) and total phenolic content (214–264 mg GAE 100 mL^{-1}) were higher in fruit juice containing immobilized cells at 4 ℃ for 4 weeks. In a study by Ding et al. (2008), the effects of storage time and microencapsulation methods on the viability of probiotic bacteria in apple and orange juices were investigated. The effect of using probiotics on physical changes in fruit juice was compared with the control groups. As a result of the study, it was observed that the microencapsulated probiotics showed higher viability than the free probiotics, and in general, an increase in pH and a decrease in °Brix concentration during the storage of probiotic juices was observed.

One of the current strategies ensure the probiotic viability is ultrasound application. Ultrasound is expanding technologies, particularly high-intensity ultrasound (HIUS), which has been thoroughly researched for food processing, primarily with regard to liquid foods (Guimarães et al., 2019). Ultrasound effectively activates waste metabolism by creating temporary holes in cells and affecting cell permeability. As a result, microorganisms receive oxygen and nutrients faster. Thus, microbial viability can be increased by applying ultrasound at low levels (Rahman et al., 2023). In probiotic strawberry juice produced by applying HIUS, it was stated that 2.5 minutes of HIUS application shortened the fermentation time (3 hours), probiotic viability was better in simulated gastrointestinal conditions, probiotic viability was higher during storage, and also improved other properties of strawberry juice (Mizuta et al., 2023).

Increment of health promoting effects of fermented fruits by probiotic fortification

The survival of probiotics during transit through the upper GIT, their colonization, and proliferation mainly in the human colon are key determinants of their health effects. Although initially the concentrations of probiotics in foods seem sufficient to benefit the host, some factors throughout the digestive system cause the probiotics to lose their effectiveness. These factors are low pH, bile salts and gastric enzymes. Gastric juice contains hydrochloric acid and pepsin which ensure a very low pH and the digestion of proteins, respectively. While this gastric juice is beneficial in inhibiting pathogenic microorganisms, it can be an obstacle for the preservation of probiotic viability. The reduction of probiotic survival during processing, storage, and after digestion is frequently mentioned in evaluations of probiotics (Shori, 2016). Because the health benefits of probiotic food products depend on the number of viable cells present at the time of consumption, maintaining the viability of the probiotic strain is the main challenge for the effectiveness of a probiotic food product. This is a prerequisite for achieving health benefits (Aspri et al., 2020). Figure 2 summarizes the potential health promoting functions of probiotic juices with respect to their effects as well as effects originating from their roles as probiotic delivery systems (Garcia et al., 2020).

Fruits and vegetables naturally contain high amounts of minerals, vitamins, carbohydrates, polyphenols, and are very important for human health with their high antioxidant capacity. Many studies have revealed the antioxidant properties of natural products used to treat various diseases such as hypertension, cancer, Alzheimer's disease, diabetes, and Parkinson's disease (Guan et al., 2021). These components, which are naturally present in the structure with fermentation, undergo various biochemical changes, improving the properties of foods and increasing their nutritional value (Tresserra-Rimbau et al., 2019). With their mechanism of action, LABs, microbial enzymes and activated endogenous plant enzymes change the structural properties and provide the release of phytochemicals (hydrolyzed polyphenols and glucosinolates, peptides, secondary metabolites, short chain fatty acids and

exopolysaccharides) known for their antioxidant, anti-inflammatory and anticancer properties. They make the consumption of fermented products more attractive. During fermentation, the activity of phytase enzyme may increase with the increase of acidity in the medium. Thus, the bioavailability of phytic acid, which is effective on the absorption of minerals, can be inhibited and its bioavailability can be increased (Leitzmann, 2016; Septembre-Malaterre et al., 2018; Rastogi et al., 2022). Consumption of probiotics is considered to have beneficial effects in the prevention of various diseases (Kandylis et al., 2016). Previous studies suggested that single or multi-strain use of probiotics in fermented beverage production might reduce blood pressure and hypertension as well as blood cholesterol levels (Khalesi et al., 2014; Sun & Buys, 2015) and can be good to fight against gastrointestinal disorders (de Oliveira et al., 2021) and to strengthen the immunity against COVID-19 virus (Singh & Rao, 2021).

Some probiotic LABs produce exopolysaccharide (EPS) and GABA (γ-aminobutyric acid) with various functions in the organism. EPS is a bacterial polysaccharide with potential effects to provide stability to food products. EPSs increase the viscosity in fruit juices and stabilize the structure, while helping probiotics to colonize in the gastrointestinal tract. It is also known to have antioxidant, antibacterial and anticancer properties (Korcz & Varga, 2021). GABA (γ-aminobutyric acid), which acts as a neurotransmitter in the central nervous system, is an amino acid group compound that controls hormone secretion, has potential antidepressant properties and calming effects (Garcia et al., 2020; Szutowska, 2020). By producing these key health promoting metabolites during fermentation as well as their delivery period, probiotics can act as key components of the juice systems which also favors their consumption from the consumer's side. The role of probiotics might rely on specific LAB species. For instance, many health benefits of *L. casei* and *L. plantarum*, which are frequently used in fruit and vegetable fermentation, are known to have probiotic properties. *L. casei* has various probiotic properties, including lipid-lowering, immunomodulatory and antioxidant properties, while *L. plantarum* colonizes the intestine effectively and improves the intestinal flora. It also has high probiotic properties in terms of regulating cholesterol and blood lipids (Khalesi et al., 2014).

Conclusion

Nowadays, plant-based food products especially fruits are at the forefront with their evaluation as fermented systems due to their valuable nutritional profiles. Fortification of these products with probiotics has become a strategy to develop fermented juices containing physiologically active numbers of probiotic cells during their shelf life. Both strain specific and intrinsic factors originating from the raw materials of the juices as well as process conditions are determinant factors for the successful development of fermented juices and delivery of the probiotics. Probiotics can also trigger the transformation of various phytochemicals during fermentation and delivery, they also produce certain metabolites such as EPS and GABA which can both improve the potential health promoting functions of these products. The survival of the probiotics in the juice matrix can be challenging and adaptation of the probiotics to the juice environment as well as development of certain encapsulation techniques should be applied. Future works should focus on the assessment of probiotic characteristics of specific LAB and FLAB strains from fruit environments to meet the increased demand of the food industry for functional probiotic juices.

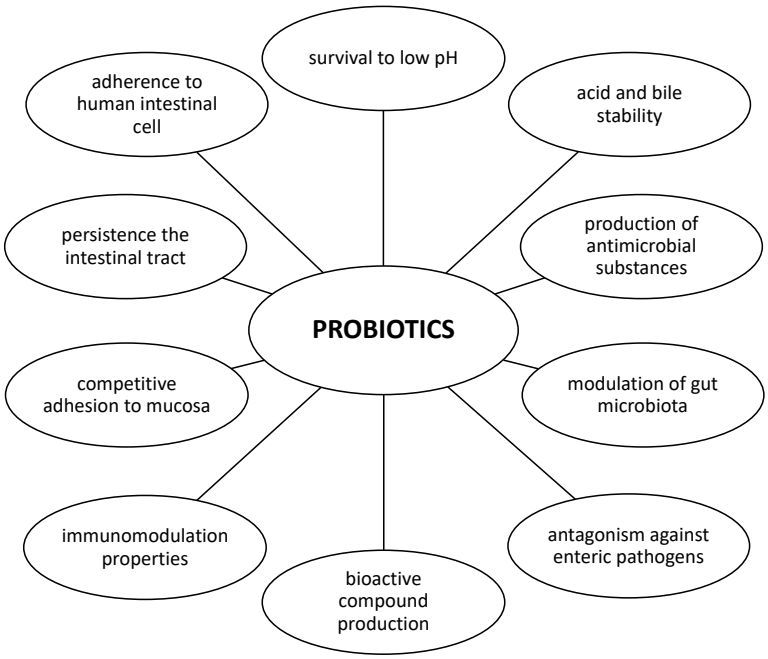

Figure 2. Health-promoting probiotic effect of fermented fruit juices and their roles as probiotic delivery systems (Garcia et al., 2020)

Declaration of competing interest

All authors declare no other competing interests.

Data availability

No data was used for the research described in the article.

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An Assessment of Forestry Policy in The European Union, Türkiye and Various Countries

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ABSTRACT

In many parts of the world, forests have been seen only as an economic value and forestry policies have been in this direction. Later, when forests started to disappear, the protectionist period started and forest policies were shaped in this direction. In this study, the reflections of sustainable foresty policy and environmental and forest protection in the Eurpoion Union (EU), Turkey and a few other countries are examined and the protection measures and recommendations of the countries are analyzed. The aim of the study is to reveal the devolopment process of forestry policies and to reveal what has been done to ensure the protection of forests. When we look at the policies followed by the countries, it is seen that the world is now pursuing a conscious forestry policy.

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Introduction

Forests have been an important resource for human beings to sustain their lives since creation. In addition to meeting their basic needs such as food, shelter, protection and heating, people have benefited from forests in terms of providing economic value in the future. These basic needs and the desire to earn money have left forests in danger of extinction over time. Forestry policies have been developed to prevent the destruction of forest. Forestry policy is the process that plans forestry in order to meet the needs of society for forest products and services. After determining the objectives of forestry policy, it investigates how these objectives can be achieved (Gümüş, 2014). Looking at the history of initiatives abd developments related to taking forestry forward, it is seen that a planned forest management first emerged in Europe (Birben, 2008). lf the history of forestry in our country is examined, it is seen that foresty started in the Ottoman period.

In this study, the devolopment of forestry policy in Turkey and the EU is analyzed and development of forestry policy of different countries is examined. In addition, issues such as protection of forests, increasing their area, rural development, trade in forest products, global warming, etc. were examined. Articles related to this subject in Turkey and in the world have been examined and synthesized.

Forestry Policies Development Process

Ottoman State Period

During the Ottoman period, the majority of the population lived in rural areas and the people had free access to forests. There were restrictions on the utilization of forests, which were only used to meet the needs of the palace, shipyard and artillery. Following the westernization movements of the Ottoman state in 1839, the first foresrty organization was established in İstanbul to protect forests (Gümüş, 2014). Forests gained an economic dimension with the outbreak of the Crimean War and the advice of the French in order to pay foreign debts due to the war. As a result of the developing bilateral relations, French expert foresters were brought to the country (Kılıç, 2004; Birben, 2008 & Gümüş, 2014). The expert foresters prepared a forestry regulation, which is the first and most important document that determines the forestry policy for the protection and operation of our country's forestry (Birben, 2008). On the one hand, The Ottoman Empire tried to protect the forests by establishing the Forestry School, the General Directorate of Forestry and the Forestry Provincial Organization, and on the other hand, it sold the forests rich in the timber through tenders (Erdem & Başkan, 2016). In 1840, the Directorate of Forestry under the Ministry of Commerce was established and a draft law was enacted to protect forests (Karabulut, 2021 & Keskin, 2010). Although it did not contain detailed

provisions, the Land Ordinance of 1858 included articles on the use of forests and coppices. Some of these provisions paved the way for forests to be cut down and turned into fields (Karabulut, 2021; Koç, 1999; Özer, 2020; Köprülü, 1949; Cin, 1978 & Kutluk, 1948). According to the Forestry Regulation issued in 1870, forests were divided into four groups as forests belonging to the State, forests belonging to foundations, forests beleonging to towns and villages and forests belonging to individuals (Dönmez, 2020). Due to the inadequacy of the Land Code in forest policies, new searches began. Louis Tassy was one of the most important names behind the regulation. Tassy prepared two important texts on the subject. First, in 1861, a 38 -article charter was prepared. With this layiha, the rules to be applied to those authorized to cut trees from the state forests through favoritism, privilege and undertaking were determined. Later in 1862, the Forestry Layiha, consisting of 4 parts and 68 articles, was prepared, which determined the management of miri forests and the duties and classes of forest officers. With the last layiha, the penalties for forest abuses were also determined (Akagündüz & Nizamoğlu, 2021).

In 1908, during the Second Constitutional Monarchy period, efforts were made to ensure that the forests were managed in a good way, and although some laws were enacted, they did not yield positive results. In 1920, the government of the Grand National Assembly of Turkey inherited from the Ottoman Empire a heavily degraded forest estate, forest contractors who exploited forests for their personal interests and a society that did not understand the benefits and importance of forests (Gülen & Özdönmez, 1981). With the Regulation, the period of unauthorized utilization of miri forests came to an end. The process of managing the forestry of the Ottoman period with technical methods and from a single source was initiated and Miri lands gained a status in the eyes of the state. Compulsory utilization of forests was legalized and controlled, the period of unlimited use was ended, and a penalty system was introduced for unauthorized use of forests. Miri forests were tried to serve the commercial objectives of the state (Koç, 2008).

In 1917, with the influence of German and Austrian forester experts who came to the country, the Law on the Management of Forests with Scientific Methods was enacted. With this law, the obligation to manage state forests according to management plans was introduced. However, the conditions of World War I and then the War of Independence made the implementation of this law impossible (Erdönmez, 2020).

Forestry experts from Austria stated that the condition of the forests in Turkey was not good, but the condition of those parts of Anatolia that had been protected from human destruction was quite good. The experts stated that there was no reliable data on the quality of the forests and according to estimated figures, the amount of forests in the Ottoman Empire was 7 million hectares. The forests were divided into 88% miri, 6% private, 3% vacant, 2% village and town coppices, and 1% land of evkaf. Assuming that every wooded area is called forest, it is stated that only 20% of the woodlands are fully forested. Experts state that if it is assumed that the amount of new trees grown each year is 3,5 m^3, the timber obtained in the Ottoman Empire each year is 24.000.000 m^3 (Karabulut, 2021).

Republic Period

Most of the regulations on forestry in the first years of the Republic were the implementation of policies that were on the agenda in the 1900s but could't be implemented due to wars. In 1920, the Coppice Law was enacted and it was decided to give 18 acres of coppice per family to villagers living at a maximum distance of 20 kilometer from forests. With this law, it was aimed to remove the problems between the state at war so that it could fight together with its nation (Sarıbey & Haykıran,2023). Although this law intended to meet the wood needs of the forest villagers, the villagers turned the areas allocated to them into fields and caused the destruction of a large part of the country's forests.

In 1922, the Law on Permission to Add Timber Meccanen came into force. According to this law, timber deemed necessary to repair the damage caused by natural disasters, insurrection and war was allowed to be obtained free of charge from state forests with a report issued by the Ministry of Economy (Yurtoğlu, 2023). In 1924, during the transition from the Ottoman land system to the Republican land system, forests on miri land were transferred to private ownership (Şimşek, 2023).

The management of forests was linked to management plans with Article 1 of the Law No. 504 on the management and Operation of Turkey's Forests with Scientific Method, which came into foece in 1924. With this approach, forests gain an economic content. With the binding of forests to the management plan, the understanding of regular operation prevails. The purpose of management is to ensure continuity in the forest and to regulate the revenue. However, the state was only satisfied with the management plans and left the forests in the hands of contractors (Gümüş,2018).

The most important regulation in the transition to technical forestry in the Republican period was the draft forestry law prepared in 1926 but not enacted. Law No. 3115 formed the basis for Laws No. 3116 and 4785, and the transition of forests to state ownership and state control in private forests were ensured (Birben, 2008). The economic depression that affected the whole world in 1929 also affected Turkey. Since there was a decline in agriculture, there was a decrease in growth rates. These factrors also negatively affected Turkish forestry (Gümüş,2018 & Pulatoğlu, 2021).

In 1927, the state forest revenue was recorded as 1.450.156 liras, while in 1929, forest revenue reached its highest level with 1.982.697 liras. After this year, the revenue values started to decrease. As the effects of the World Economic Crisis began to disappear, the forest revenue value only increased to 1.830.000 liras in 1936 (Yurtoğlu, 2023). In the 1930s, the policy of statism started to be implemented in the economy.

In 1937, the state forest management system was introduced. In the same year, the Forestry Law No. 3116 entered into force (Polatoğlu, 2021). The law stipulated that a place must be at least 5 hectares in size in order to be a forest. With this provision, areas smaller than 5 hectares were excluded from forest status and the expansion of forest areas was prevented. The lack of adequete infrastructure for the implementation of the law has increased forest destruction. Peasants and forest traders who lost their right of access and the declarations of forest

traders to change the law caused the need for wood to be met too much and too quickly, so the forest were rapidly destroyed. In 1927, the amount of forested area covering our country was 18%, while it decreased to 10% in 1938 (Benli, 2014).

A local newspaper, Bartın Newspaper, announced the forestry policies to the public. After reading the new regulations in the newspaper, the people thought that they would not be able to meet their wood needs from the forests, so they cut too much from the forests and stored enough wood in their homes for maybe 10 years. Some of the news in the newspapers were perceived as warnings by the General Directorate of Forestry and new regulations were made in line with these warnings. In the period from 1937 to 1957, different forestry practices and policy changes were made with the regulations made in Law No. 3116. As the practices in this period formed the basis of the Republican Period forestry, we can say that their effects have continued until today (Atmış & Gençay 2014).

During World War II, various laws were enacted on tree communities and legal gaps related to forests were tried to be eliminated. With the effect of the war, while trying to meet the country's need for wood raw material, new species such as eucalyptus and sweetgum trees were started to be planted. In addition to the felling of forests to obtain wood, fires set due to the war and for land acquisition caused a decrease in forest areas. However, during the war, railroad construction, road construction, telephone lines and power plants were constructed and fires were intervened in a timely manner, preventing the waste of timber and reducing costs (Yurtoğlu, 2021).

During the multi-party period, many legal arrangements were made regarding forests. For example, in 1946, Article 7 of the Law No. 3444 supplementary to the Forestry Law No. 4914 was amended, stating that the forests operated by the Forestry Enterprises and their activities for their revolving capital shall be operated according to the principles and commercial justifications to be determined by the Council of Ministers, not according to the laws of auction, auction, general accounting and tender. The operating balance sheet of these enterprises, together with the final accounts, shall be submitted to the Court of Accounts within 7 months as of the end of the year. The Court of Accounts conducts its examinations by looking at the commercial structure and legislation of forest enterprises. Law No. 4920 and the first article of the Law on the Exemption of State Forest Enterprises from Certain Taxes exempted the tree communities belonging to the General Directorate of Forestry from taxes. The articles of many other laws were amended during this period. In addition to afforestation activities during this period, nurseries were established in various places and the need for saplings to be used in afforestation activities was met from these nurseries. In addition, in order to combat smuggling and fires, one of the biggest problems of the country's forests, guards and watchmen were employed and watchtowers were built. Thus, the opportunity to intervene in fires and illegal logging in larger forest areas increased (Yurtoğlu, 2022).

In the following periods, the development of technology, change and increase in human needs led to policy changes in forestry. This period is characterized as the modern forestry period.

Contemporary Forestry Period

According to a widespread belief in our country, forestry is not a practice characterized as logging or lumbering. Modern science has shown that such characterizations are an important misconception. It has proven the multifaceted effectiveness of forestry for societies (Pehlivanoğlu, 1979).

Modern forestry has brought a new perspective to forest resources that depend on biological qualities. Foresters' need for socioeconomic knowledge such as management, planning, business, mathematics and economics has increased (Daşdemir, 1999).

The European understanding of forestry, which imitated nature and prioritized the wishes of the society, has expanded in the USA and transformed into a new system based on demands, multifaceted utilization and intervention in nature**.** This sector has a very different importance from Europe in terms of the allocation of very large forest areas to various economic activities, watershed and management, and the conversion of accumulated wealth into liquidity. In addition to the principle of continuity that Europe brought to forestry, the United States has incorporated the principle of multifaceted utilization on a universal scale. The principle of multi-directional utilization brought a new scope to the practice and planning in forestry and the biological orientation of the forest, and new analysis techniques were introduced to forestry (Geray, 1989).

Modern forestry is a sustainable work that covers all economic, sociocultural, managerial, biological and technical studies carried out with the aim of providing goods and services to the society by systematically utilizing forest resources (Daşdemir, 2016). In addition, after a large part of the world's forests were plundered, it was realized that forests produce many ecological values (Türker et al.; 2002).

Forest Law No. 3116, which pioneered the transition to modern forestry in our country, was replaced by Forest Law No. 6831 in 1956, which is still in force (Gümüş, 2014). Forest Law No. 3116 is the first Forest Law that considers the real and ideal values and functions of the forest as a national asset, wants to ensure the establishment and development of high quality forestry enterprises, and aims to increase the quality and quantity of forest assets (İnal, 1964). Law No. 3116 introduced state forest ownership and management in forestry. Forestry directorates were closed and replaced by "Forestry Directorate of Translation", "Forestry Inspectorate" and as of 1945, "Forest Management Directorates", which is the current structure, were established (Özden, 2019).

Forests are strictly defined by law to be operated by the state. Resources that can be exhausted or renewed are subjected to the pressures of economic developments due to the increase in human population and developing technology. For this reason, continuous forest management has been abandoned in many countries and a system of progressive forest management and cultural forest management has been adopted. With this system, a form of management that can be grown by human hands according to industrial needs and requires economic planning has been introduced. Considering that the world's resources have an international value, we can reveal the importance of resource management of countries (İstanbullu, 1974).

Until 1961, the constitutions enacted did not include provisions on forestry. However, the fact that forest-public relations were not regulated and the provisions of the law were not enforced made it impossible to protect forests and led to an increase in forest crimes. In addition, the use of forests by political parties in their propaganda, the frequent amendment of forest laws by the ruling parties and the enactment of new laws to provide amnesty for forest crimes have revealed the necessity of constitutional security for the protection of forests (Akar & Tolunay, 2018).

The provisions on the protection of forests were first secured by the 1961 Constitution. The 1961 Constitution included some provisions on the protection of forests together with the principles that the development and protection of forests, supervision and control of forests is the duty of the state, and forest management is under state control. However, this approach could not be maintained for a long time. Article 131 of the Constitution was amended by Law No. 1255, which entered into force in 1970. With this amendment, approaches and practices that will cause the destruction and reduction of forests in our country have become a constitutional provision (Erdönmez & Yurdakul Erol, 2021).

In the 1982 Constitution, Articles 169 and 170 become an important basis for the protection of forests. Paragraph 2 of Article 169 states that "The ownership of State Forests cannot be transferred. State forests are managed and operated by the State according to the law. These forests cannot be acquired by prescription and cannot be subject to easement rights except for public benefit." When the Forestry Law No. 6831 is analyzed, it is seen that the law contains regulations on the use of forests for non-forestry activities in fourteen articles. Three of these articles include regulations on the granting of permits from forests, one on the leasing of existing facilities, one on their use as recreation areas, and one on the regulation of the establishment of easement rights (Olgun & Tolunay, 2018).

The Forestry Law No. 6831, which is a special law and also regulates the penal provisions, is evaluated together with the Turkish Penal Code system. Since the amendments made in the Criminal Law or Laws directly or indirectly affect the Forest Law, they also cause amendments to be made in the Forest Law. For example; from Article 70 to Article 116 of the Forestry Law No. 6831, a total of 33 articles related to penal provisions have been amended. Some of the amendments have been radical changes in terms of the penal provisions of the Forestry Law and have caused significant changes in the judiciary and forestry practices (Elvan, 2009).

Factors Affecting Forestry in Turkey at National and International Level

Founded in 1958, the European Economic Community (EEC), which initially included 6 countries in Europe, was later renamed the European Union (EU). The EU is a common market established to create economic cooperation between member states. After 1973, other countries started to join the community. Turkey became a candidate member of the European Union (EU) with the signing of the Ankara Treaty in 1963 and applied for full membership in 1987 (Haliloğlu & Tolunay, 2009; Yıldırım & Budak, 2010 and Atmış & Gençay, 2014). The European Union held a Summit in Helsinki on December 10-11, 1999. In this process, Turkey was accepted as a candidate country to the EU. Following the preparation of the Accession Partnership Document by the EU Commission, Turkey announced its National Program. Within this framework, our country has started to work on this issue by committing to organize its legislation in the field of forestry according to the EU legislation (Dölarslan, 2007).

In the late 1970s, the European Union drew attention to the fact that environmental pollution, combating forest fires and deforestation issues related to forestry were on the agenda of the union (Yurdakul Erol; Akgün, 2005) and gave importance to the protection and increase of forest areas by giving up seeing forests as a trade commodity. It has also started to take measures within the framework of global warming and climate change. For this reason, they have tried to promote the use of biomass in order to ensure efficient and smart utilization of forests, to prevent the waste of wood and forest products, and to increase the use of renewable energy sources and recycling (Ciccarese et al. 2014).

The main objective of the EU forestry strategy is to strengthen sustainable forest management as stated in the Forest Principles and defined in the Ministerial Conference. For this purpose, it is stated in the strategy that active participation in all international transactions is valid for the forestry sector (Velioğlu & Yıldırım, 2007).

Although the developments in the field of forestry are closely followed in EU member states, forestry has not been an independent policy branch in the EU. It has only been recognized as a sub-branch of policy branches such as agriculture and environment. Although there are some specific regulations on forestry in the EU Acquis, a detailed common forestry policy has not been established (Özcan, 2008). In 1992, the Principles of Forestry adopted at the end of the United Nations Conference on Environment and Development held in Rio de Janeiro, Brazil, made an impact in the EU and forestry gained a different dimension after these documents. The aim of the conference is to ensure that sustainable development is adopted as a global understanding (Orman Genel Müdürlüğü [OGM], 2020). Sustainable Development is a development model that can meet the needs of today's people without depriving our future generations of the right to meet their needs (Rahmanlar, 2016).

Under the umbrella of the United Nations (UN), the Intergovernmental Panel on Forestry (IPF), the Intergovernmental Forestry Forum (IFF) and most recently the United Nations Forestry Forum (UNFF) were established and 270 decisions agreed upon by all member countries to be implemented at the global level were taken and grouped under 16 headings. In order to implement the 270 decisions taken under UN supervision, the world was divided into nine regions. Turkey is included in both the Pan-European and the Near East Process. The criteria do not vary much from country to country. For this reason, six criteria for sustainable forest management have been determined by a global consensus. These criteria are as follows: forest resources, health and vitality of forests, conservation functions of forests, socio-economic functions of forests and biodiversity. In some regions,

political, legal and institutional framework criteria are added to these criteria and classified as seven items (Erdoğan, 2010).

An independent forestry policy has not been established in the EU. In 1998, the forestry strategy prepared to determine the status and objectives of forestry and forests within the EU and the obligations of member and candidate countries was an important step in establishing a common forestry policy of the EU. The EU Forestry Strategy, made in the light of common decisions, will be the basis of a common forestry policy in the future (Yıldız & Atmış, 2014). In addition to vital activities such as regulating the water regime, protecting the soil and preventing environmental pollution, which are of great importance in the life of society and the environment, the role of forests in the protection of biodiversity is also extremely important. Our country has an important potential in terms of "biodiversity" and "protected areas", which are becoming increasingly important both at national and global level. Protection of biodiversity and development of protected areas both in terms of area and functions are among the main objectives of the sector (Başbakanlık Devlet Planlama Teşkilatı [BDPT], 2007).

Among the EU's core principles on forestry; Ensuring the proper functioning of the EU forestry sector in rural areas as well as the sustainable development of forests for their conservation, mitigating the effects of climate change, protecting forests by preserving biodiversity and restoring degraded forests, reducing exposure to abiotic and biotic factors, developing Sustainable Forestry Management (SFM) in the EU economically, socially and ecologically, protecting the environment and forest assets as well as erosion and soil protection, water management, carbon storage and air quality improvement, development of forest monitoring tools and equipment within the framework of environmental agreements, ensuring competition in the EU forestry sector depending on industries, increasing the use of environmentally friendly wood and other forest products, increasing the SOY, certification and labeling of related products, contributing to the EU's development policy by ensuring sustainable management of forests as one of the ways to reduce impoverishment (Özcan, 2008).

EU trade policy states that it will ensure full implementation of biodiversity provisions included in all trade agreements, further assess the impact of trade agreements on biodiversity and work to strengthen biodiversity provisions in new agreements. An agreement was reached between the European Parliament and the Council in December 2022 on the Draft Anti-Deforestation Regulation on deforestation-free supply chains prepared by the Commission. According to the Regulation, traceability is a prerequisite for entry into the EU market, especially for products such as palm oil, soy, cocoa, coffee, cattle, rubber and timber. It is aimed to ensure that the products within the scope of the legislation do not cause deforestation for entry into the EU market, that they are produced in accordance with the legislation of the relevant country and that the obligation to show that the products are produced in a way that does not cause deforestation is fulfilled. At the international level, the EU will support sustainable agriculture and fisheries practices to protect and restore the world's forests, paying particular attention to sustainable water resources management, restoration of degraded landscapes and biodiversity conservation (URL1, 2023).

The EU forest strategy is based on the European Green Deal and the EU 2030 Biodiversity Strategy. It is expected to contribute to achieving a greenhouse gas emission reduction target of at least 55% by 2030 and a climate neutral EU by 2050 (URL2, 2023). In this framework, it aims to increase the contribution of forestry and forest-based values in order to increase and protect the welfare of prosperous rural areas by achieving a sustainable and climate-neutral economy by 2050 (Aşan, 2023).

National development plans determine strategies for the development of the forestry sector and ensure their realization. The latest development plan (12th Development Plan) covers the years 2024-2028. According to this plan, it is aimed to increase the forest wealth by managing forests in accordance with sustainable forest management criteria and indicators, taking into account international conventions and national commitments, including the United Nations Global Forest Goals and Rio Conventions. For this purpose, forests will be established with species resistant to climate change and management plans will be developed to increase sink areas. By reducing deforestation, biodiversity and water resources will be protected and their contribution to energy, health, food and tourism sectors will be increased. Landslide, flood and avalanche control projects will be implemented to combat erosion. Carbon and green certification activities related to forestry will be developed. Recycling of forest products will be ensured. Compliance of forestry statistics with world standards will be ensured. Organized industrial zones will be established in regions with dense forests. International trade of forestry companies in Turkey will be supported. The share of the sector in the economy will be increased. Certification procedures will be increased throughout the country to facilitate the trade of forest products. Industrial afforestation activities of the private sector will be supported. The use of wood in workplaces and residences will be increased. Price stability will be ensured in forest products. Development of forest villagers will be ensured and migration from villages to cities will be prevented. Measures to prevent forest fires will be increased and effective response capacity will be improved. Buffer zones will be established in sensitive areas by using fire resistant species (Onikinci Kalkınma Planı, 2023).

In Turkey and in many other countries of the world, forestry was seen only as an economic activity. The first thing that came to mind when forest and forestry were mentioned was timber and firewood production. For this reason, forests have been destroyed for many years and even their amount has decreased day by day. The transition from consumption forestry to conservation forestry took time. However, recently, globalization and environmental protection have been among the most important issues on the world agenda (Yıldırım; Budak, 2010). Environmental problems and deforestation have gained a global dimension and international solutions have been sought. The EU is a very strong and effective organization on environmental problems. EU member countries have to be a party to the environmental conventions to which the EU is a party and they have to bring their countries into compliance with the terms of the conventions in order to fulfill the requirements

of these conventions. Otherwise, those at the membership stage will not be able to become full members and full members will face the danger of having their funds cut off or being prosecuted in the courts.

Turkey is striving to join the EU because it wants to be economically and politically strong in its geography. Turkey is a productive power with a competitive advantage in economic and trade compared to other members of the EU. The Turkish economy has made a certain economic progress and then stabilized. Turkey's trade potential will contribute to its full membership of the EU. In this way, as a result of mutual trust and views in trade, management and technical problems that occur in market conditions can be eliminated (Akyüz et al., 2010).

In July 2016, the European Commission (EC) transformed land use, land use change and forestry into the 2030 Climate and Energy Framework for greenhouse gas emissions. As part of the Paris agreement, the Climate and Energy Framework aims to reduce total emissions by 40% by 2030 for all sectors. Sustainable forestry management has become climate-oriented. Reducing the impacts of climate change is the main goal and forests have started to be considered as carbon sinks (Nabuurs et al., 2017).

On average, there are 215 million ha. of forests and woodlands on the European continent. This number corresponds to 30% of the total area. There are differences between forest areas in terms of climatic, ecological, geographical, socio-economic conditions. In the whole of Europe, the EU's forest area is around 130 million hectares. This amount corresponds to 35% of the Union. After the recent enlargement in 2004 with the accession of new member states, the forest area of the Union has increased by about 7 million hectares. On the other hand, the species diversity of the Union also varies among member countries. There are even differences between countries in terms of ownership. The difference in ownership stems from cultural and historical differences. In 15 member countries of the Union, privately owned forests constitute 65%. However, public ownership is dominant in forests in 10 of the countries that became members after 2004 (Yurdakul Erol & Akgün, 2006).

As environmental awareness is gradually increasing in our country, the legislative infrastructure is also being developed rapidly. In this context, the high potential of renewable energy resources in our country provides an advantage. The fact that environmental legislation is very comprehensive is among the factors affecting Turkey's harmonization efforts since it concerns not only the present but also the future of the society (Turan Bayram et al., 2011).

Discussion

Forestry activities were first seen in China around 400 BC. During this period, studies were carried out on how to ensure a continuous supply of wood for a long time. In Sri Lanka and India, rules on the control of hunting and logging and the management of forest resources began to be established about 2000 years ago. In Western countries, on the other hand, protective measures began to be taken much later. For example, it was discovered in the 13th century in England that the reason for the decline in hunting animals was due to the decrease in the presence of forests. In Switzerland, a legal regulation was enacted in 1343 to protect forests in order to ensure a continuous supply of wood and to protect against avalanche disasters. In Germany in the 16th century, deforestation was tried to be prevented by limiting the supply of wood and imposing sanctions to prevent the use of wood (URL3, 2023).

Forestry activities in our country started earlier than many countries with the influence of Germany and France. Forestry organization has been continuing its duty since 1839. Many regulations have been made until today in order to protect, develop and ensure the sustainability of forests. The regulations have been secured by laws. In addition, public participation is among the management strategies to ensure effective management of forests.

The concept of management has two meanings. The first means to direct and manage, while the second refers to the organization that operates an activity and all its offices and employees. The function of management is to organize and carry out forestry activities. The most important function is the sustainability of management. Sustainable forest management can be defined as a management that ensures the integrity, productivity, rejuvenation capacity, biodiversity, ecological, economic and social benefits of forest areas and resources today and in the future, at national and global level, in a sustainable manner for the benefit of society and that does not harm other ecosystems. Participation is the development process between local people and development officials in which the target groups are the guides and even determinants in analyzing the situation for solving problems, planning, implementing and evaluating development. Participatory management means project design, implementation, supervision, evaluation, correction, cooperation with the affected people and supporting institutions (Velioğlu & Yıldırım, 2007). Local community participation in forest management and forest ownership is increasing. However, forest-based communities have overridden some of the positive effects of increased participation on ownership. Instead of supporting poor and indigenous people, forestry and regulatory policies favor access to forests for the rich so that they can use forest resources for urban needs. This leads to negative discrimination between the poor and the rich. However, retaining forest benefits locally can provide options for improved prosperity in these areas. Indeed, the great commercial and livelihood value of forests in poverty reduction has attracted attention (Larson & Ribot, 2007). The fact that poor people see forests as a livelihood asset and rely on forests for most of their vital needs has led to the emergence of social forestry. However, at first it was perceived only as the sale of wood to meet the need for wood, but over time it has reached its current evolution (Hobley, 2005).

As an institution intertwined with the public, the General Directorate of Forestry carries out various activities within the scope of participatory management policies. The most important of these is the training of volunteers from the civilian population in extinguishing forest fires. In addition, trainings on sapling planting are also organized. Thus, by ensuring the participation of the public, we raise awareness of our institution and the work we do, as well as raising individuals who are helpful to our organization.

Public participation must be ensured in environmental protection activities, problem solving and rural development. The basic plan of rural development is to increase the living standards of rural people by increasing their income. Rural development is a problem in Turkey as in many parts of the world. In the EU, the problems related to rural development are gradually coming to light. The EU is constantly making program and policy changes in an effort to create financial resources to solve the problems. Process changes are also closely related to our country in the EU membership process. Rural development policies in Turkey have generally consisted of projects. Emphasis has been given to agricultural production and marketing with practices supported by external resources (Gülçubuk ve Karabıyık, 2002). In addition, the relationship between forest resources and forest villagers makes rural development a privileged issue in Turkey. Rural development has a critical importance for forest villagers. Because forest villagers are the group with the lowest income level as well as low education, health services, infrastructure works and social opportunities (Yurdakul Erol & Yıldırım, 2017). Therefore, forest villagers have caused pressure on forests in terms of the use of forest resources and land. They have made a large amount of clearing from forests to use the land as agricultural land. In the following periods, with the cadastral law, the qualifications of such places changed and they became fields. Forests remained dense in hilly and roadless areas. After the forests were put into operation, the need for forest road construction arose. In our country, the general lines of forest roads were completed in 1979. However, an average of 1000 km of roads are constructed every year. The standards of forest roads in our country are similar to the standards of Austria, one of the EU member countries (Çağlar & Acar, 2009). With the opening of roads, production has increased and forest products have contributed to the increase in trade volume.

Within the scope of rural development, the Forestry Organization supports rural development by producing various afforestation and non-wood product cultivation projects, as well as providing jobs for forest villagers to gain income, producing various afforestation and non-wood product cultivation projects, providing cattle and sheep loans to villagers through Orköy projects, solar energy systems for houses, renewal of electrical installations, and providing drums and tractors. In addition, the increase in forest revenues has increased the share transferred to the treasury up to 15 percent.

Apart from European forestry, the forestry policies and strategies of some countries in Asia, Africa and South America: In Ethiopia, the development of forestry policy is intertwined with the evolution and conscience of the state structure. An organized state structure in Ethiopia emerged after World War II. The forestry policy process first emerged under the Italians. However, the Italians were expelled before they could introduce forestry policies. The first forestry law was published in 1965. An autonomous forestry sector came after the mid-1970s. The famine and drought of 1985 turned the country's attention to forests and environmental problems. Since the 1990s, with the impact of economic growth, both forestry and environmental protection problems have been focused on (Ayana et al., 2012).

During the war years, as in many other countries, the number of forests tended to decrease in our country. Later, thanks to the measures taken and the policies followed, the forest presence has increased until today. The forest presence, which was 6 million hectares at the beginning, reached 10.5 million hectares in 1949 (Evsile, 2018). It was recorded as 20.2 hectares in 1973, 20.8 hectares. in 1999, 21.2 hectares. in 2004, 21.7 hectares in 2012, 22.3 hectares in 2015, 22.9 hectares in 2020 and 23.1 hectares in 2021 (URL4, 2024).

After the deterioration of forestry management in Nepal, the forest-people relationship was managed by the government by making laws. The law-making process was not based on the opinions of the people and non-governmental organizations, but only on the opinions of parliamentarians. Decreasing forest areas started to increase again with the harsh laws of the government (Ojha et al., 2007).

In general, forestry management and policies in our country are under the influence of political parties and governments. Governments can cause the destruction of forests by amending laws and regulations. For example, the 2B law paved the way for the clearing of forests. With the opening of forests to sectors such as mining, construction and tourism by introducing easement rights, the destruction has increased.

Forest areas are very low in Bangladesh. However, forests are of great importance for the livelihood of local people. Forest management in Bangladesh started in 1864. 60% of the forests in the country are state forests. This is one of the countries that will be most affected by global warming and climate change. In 1989, forest laws tried to prevent deforestation by providing stricter penalties. The actions were based on forest protection but did not support social forestry. In 2000, a new law tried to adapt to social forestry (Alam, 2009).

In our country, 99.9% of forest ownership, almost all of it, belongs to the state. While forest protection is supported by law, rural and social forestry policies are prioritized in order to contribute to the livelihood of the people living in rural areas. At the same time, these policies prevent the destruction of forests.

Brazil's tropical forests experienced a major forest loss between 2000 and 2012, with forest cover loss reaching 32%. Increasing population and consumer demands will increase pressure on forests. Balancing increasing demand for different land uses, reducing competition for land, increasing biodiversity conservation and improving ecosystem services could be a potential solution. Increasing agricultural productivity could accelerate deforestation. The development of cattle ranching can contribute to the development of forests (Alves-Pinto et al., 2016).

In our country, forests are being destroyed to gain agricultural land. In addition, grazing in forests due to the insufficiency of grasslands and pastures also causes the destruction of saplings. Grazing plans and increasing pastures will prevent deforestation.

Approximately 82 percent of forests in Poland are managed by the state. Forest area is 9.483.000 ha. Looking at the history of forestry in Poland, it has been determined that the increase in the demand for wood has decreased the forest presence. The products obtained from forests are

generally industrial wood. In 2021, 42.2 million m³ of wood was produced. 97 percent of the forests have FSC certification. The Polish forestry law entered into force in 1991. Forestry activities started in Turkey much earlier than in Poland. In terms of forest area, Turkey has 3 times the area of Poland. The amount of FSC corresponds to 28 percent of the forests. In Turkey, it was determined to be approximately 27.7 million m³ in 2021. Although Turkey has more forests in terms of area, it is seen that Turkey's forests are inefficient when the production amounts are compared (Gedik et al., 2023).

If the political development of Turkey and some countries in the world is analyzed:

Gümüş (2014) analyzed the forestry policy from the Ottoman Empire to the present day in his study. The forestry organization of our country was established to generate income. The value of wood has had an important place in the organizations from the first establishment, which aimed to protect forests and regulate the sale of wood, to the present day. Over time, ecological concerns started to emerge and this situation started to have an impact on political approaches. With the beginning of conscious forestry in our country, the developments in forestry have been examined and it has been emphasized that there are structural defects in the forest organization today. It is said that the organization is unable to work due to unnecessary staffing and unnecessary units. In addition, it is argued that resources are wasted due to the excessive number of staff, and it is stated that the ministry should be closed and a new organizational structure should be arranged.

Birben (2007), in his research, evaluated the forestry policy of Turkey after 1937. The year 1937 is considered as the milestone of transition to technical forestry in Turkey. In this year, the Forestry Law No. 3116 came into force. The socio-economic and political development of the law was analyzed. Between 1937 and 1950, forests were tried to be protected in accordance with the principle of statism. However, it is seen that the forest laws enacted in the 1950-1960 period and after 1980 were not based on the protection function and focused only on the financial return of forests.

The forestry policy in our country was generally based on wood production during the Ottoman period and there was a lot of forest destruction due to the lack of controlled forestry and wars. During the Republican period, the destruction of forests continued for a while. In 1937, with the introduction of technical forestry in the modern sense, conservation forestry started. Today, our forest cover tends to increase.

Alam (2009) examined the historical development of Bangladesh's national forest policy in his article. In order to increase government revenues, the trade of forest resources is at the forefront. This led to the decline of forests. Later on, the extreme impact of climate change on the country made the need to protect forests evident. New laws were passed to protect forests and develop social forestry.

Ayana et al. (2012) examine and explain the historical development of Ethiopia's forestry policy in their study. The use and management of forests were designed as social and institutional arrangements. During the imperial period, resources such as timber from natural forests were seen as the main source of income to fuel the economy. The rapid depletion of forest resources raised the question of conservation. To protect forests, the Empire established an autonomous forestry organization to enforce laws and ensure forest maintenance.

Ojha et al. (2007) examined the social and historical process of forestry policy decision-making in Nepal. Forest-people relationship was managed by the government through law-making after forestry management deteriorated. In the law-making process, the opinions of the people and non-governmental organizations were not taken from the public and only the opinions of the parliamentarians were taken as basis. 15 laws were content analyzed. In general terms, the content analysis examined issues such as the government's toughening of laws and exclusion of technical staff in law-making, sale of forest products, increasing taxes, inventorying, biodiversity strategy, non-wood forest products sales policy, etc. Citizens' perspectives on these issues were also assessed. It shows that the governmental management of Nepal's forests after the 80s has had positive results in terms of forestry. In the first period, non-governmental organizations and the public were excluded from law-making, but in the second period, cooperation was ensured.

Larson and Ribot (2007) gave examples from Honduras and Senegal in their study. In both countries, as in forest policies in many parts of the world, laws are heavily skewed against local communities due to economic and social disadvantages. Forest policies create a double standard between the rich and the poor. Poor communities remain subject to these privileged elites.

Although the names of countries change, when it comes to forestry, the first thing that comes to mind is wood production and its contribution to the economy. The forestry policies of countries generally lead to the destruction of forests. The presence of quality forests has shown a tendency to decrease in many countries. With the inadequacy of the products obtained over time and the effect of climate change, forestry policies have been tried to be changed to protect forests. In our country, the protection of forests has been tried to be ensured by establishing laws. However, the laws have paved the way for the destruction of forests by granting permission easement rights from forests. In addition, with the issuance of the 2B article implementation regulation, land acquisition has been legalized by opening up forests.

As an answer to questions such as what is the place of the European Union in the forestry sector, what does the union do about forestry, what are the things that the member states of the union should do?

Gülçubuk and Karabıyık (2002), in their study, mentioned the importance and objective of rural development policies of the EU in order to eliminate the balance difference between rural and urban regions. The main objective of rural development is to improve the living standards of the rural population by increasing their income level. Rural development is an important problem in Turkey as well as in many other parts of the world. In the EU, rural development policies are planned according to economic, environmental and social needs. In Turkey, there is no outlined rural development policy and a policy needs to be developed. The country's policy should see

rural development and agricultural development as a whole. Agricultural productivity and quality should be increased. Projects should be produced at regional and local level. Financial resource and budget shortage should be solved. Rural development strategies should be developed in line with EU policies and the institutional structure should be organized accordingly.

Yurdakul Erol and Akgün (2005), in this article the development process of EU forestry policy is discussed. When EU member countries are evaluated in terms of forestry, differences between forest ownership in the countries stand out. Most of the countries have private ownership and species diversity is not very high. International policies have been influential in the formulation of EU forestry policy. However, forestry structure, biodiversity, rural development, climate change and forest industry are also influential. Important issues related to forestry in the EU are listed as participation, the relationship between rural development and forestry, protection of biodiversity, intersectoral and international cooperation, increasing the service production efficiency of forests, trade in forest products, ensuring ecosystem balance and environmental protection. In the new regulations to be made in the field of forestry in our country, it would be appropriate to take into consideration the EU forestry principles and objectives as well as the decisions taken in international meetings on forestry. Turkey is one of the countries in the process of EU membership.

Velioğlu and Yıldırım (2007), In this study, firstly the concepts of public participation in forest management in Turkish Forest Legislation and then the concepts of participation in forest management according to EU Legislation are examined. The views on participation in forestry management are mostly related to participation in the decision-making process. The right to access information and documents in participation is not regulated in the Turkish Legal System. However, it is used through interpretation.

Haliloğlu and Tolunay (2009), Turkey became a candidate member of the EU in 1963 but became a full member in 1987. Countries wishing to become a member of the EU have to comply with the conditions of the union and the agreements signed by the union. Turkey will have to adapt its forestry policies to the EU policies. Initially, forestry policies in the EU were based on lumbering because they were evaluated from an economic point of view and they did not have a proper forestry policy. With the new global approaches to forests being environmentally oriented, conscious forestry policies have been adopted. International negotiations and agreements on deforestation and global warming have begun. These processes have directed policies towards protection and development. In the reports of Turkey-EU negotiations, it was stated that our country has an advanced and good structure in forestry issues but needs to make efforts in implementation.

Özüdoğru and Duygu (2009) mentioned the advantages and disadvantages of Turkey in Turkey's EU accession negotiations. The biggest advantage of our country is the richness of biodiversity. When the Natura 2000 Network is evaluated, the protected areas of our country are 5.49% of its surface area. And this ratio is very insufficient. Special protection measures should be taken within the scope of Natura 2000. Although our natural resources and forests are in the process of extinction and degradation, we have an advantage over the EU. Because our number of wild species and endemic species is quite good. The fact that our membership process is long will also give us an advantage in terms of implementing the EU criteria.

Yıldırım and Budak (2010) discuss the changes in the environmental policies of cultural, social, economic and political life, especially in the environmental field, in the process of full membership to the EU. Turkey is seen to have made progress in terms of increasing administrative capacity and administrative authorities at the central level. EU environmental policies have an impact on the policies of countries wishing to become a member. Turkey's environmental policy will be insufficient compared to the EU environmental policy.

Yıldız with Atmış (2014) examined the similarities and differences between EU forestry strategies and Turkish forestry policies. It is seen that rural development in the EU is realized systematically and financially supported, unlike in Turkey. While the use of wood biomass is encouraged in the EU because forests are carbon sinks, in Turkey the rural population is directed to alternative energy sources other than wood. The forestry program of our country, which is in the process of full membership to the EU, should be revised to adapt to changing conditions.

Ciccarese et al. (2014) focused on the use of wood as biomass in EU Member States and its widespread use as a renewable energy source, industrialization, which mainly uses wood raw materials, and the efficient use and recycling of wood. However, increased wood utilization will increase wood market prices. Cascade system was evaluated within the scope of energy utilization. The cascade system is the re-evaluation of wood according to its added value, derivation of new uses and recycling. As this will reduce the supply problem, forest fragmentation can be prevented, climate and environment can be protected and carbon balance can be contributed. Effective use and recycling of wood should be included in EU environmental policies. The amount of wood waste should be reduced to zero by increasing the use of biomass to ensure the carbon cycle.

Alves-Pinto et al. (2016) mentioned in their article that managing land use well will ensure ecosystem and biodiversity conservation. It will also provide social, economic and environmental benefits. However, in Brazil, which has a rich biodiversity, intensive cattle breeding, which has a good economic return, shows that soil and forest lands are sacrificed. This leads to rapid deforestation. Sustainable policies should be developed to ensure the continuity of forests. Forests should be restored and sectors should be diversified to reduce pressure. Recommendations for rural development include beekeeping, development of rural tourism and increasing potential food production to protect forests and biodiversity.

Yurdakul Erol with Yıldırım (2017) considered the relationship between forest resources and forest villagers as a privileged part of rural development forestry policy. The development and protection of forest villagers and forest resources depend on supporting forests. By improving the living standards of forest villagers, their

pressure on forests can be reduced. Forest laws also support the development of forest villagers by providing some funds and granting some rights. In addition, forest laws define the protection of forests, afforestation, protection from forest fires and the inclusion of certain areas as protected areas. Basically, supporting forest villagers and ensuring rural development are among the objectives of Turkey's forestry policy. Rural development will contribute to the protection of forests.

Nabuurs et al. (2017) focused on issues such as improving forest management, expanding forest areas, energy substitution, and building forest reserves. In order to ensure sustainable forestry, which they call smart climate forestry, it has been suggested that carbon sequestration should be ensured as well as providing energy and protecting forests by considering all chains from forest to wood products. For this, abandoned agricultural lands can be forestized to reduce carbon emissions. EU member states are indebted due to carbon emissions and this should be alleviated. The EU will mitigate forestry and forest sector carbon emissions by 20% by 2050.

Conclusion

Forest policies have developed gradually over the years according to changing conditions and the needs of the time. As a result, in Turkey and in many other parts of the world, the concept of forestry policy was initially conceived only in terms of generating economic income from forests. Governments all over the world have accompanied the destruction of forests with their laws in order to generate income. In fact, in many places, laws have been enacted that do not support the people living in rural areas, but rather favor the rich. As such, forests have become extinct over time. Until the Helsinki Summit, the member states of the European Union saw forests only as a source of income. After global warming and the importance of forests came to the agenda at the summit, the EU decided to change its policies. The EU also obliged the member countries to comply with the decisions it took and the international agreements it abided by. These developments are important in terms of understanding the importance of forests. Countries have started to pursue conservation and development policies instead of pursuing consumption forestry policies. They have developed rural development and social forestry policies to protect forests. They have proposed various solutions to protect the environment and force the countries of the world to comply with the policies they have developed. In short, after understanding the importance of forests, all countries have united on this issue. The world is now pursuing a conscious forestry policy.

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Bioactive Compounds and Industrial Peeling Applications of Inner and Outer Shells of Chestnuts (*Castanea* spp.)

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ABSTRACT

The aim of this review is to provide information concerning the types of chestnut shells (inner and outer), their compositions and bioactive compounds, as well as to mention industrial peeling applications. These shells are comprised of high-valued natural active compounds, such as polyphenols (phenolic acids, flavonoids, tannins, hydroxycoumarins -scopoletin, scoparone-), pigments (melanin) and minor compounds (minerals, dietary fiber, vitamin C and E, essential amino acids and fatty acids). The total phenolic acids and flavonoid content of *C. sativa* shell were ranged between 119.17-223.62 mg/kg db and 330 – 503 mg CE/g. It is also a good source of vitamin C with reported levels of 15.57 and 28.97 mg AA/100 mg db in water and ethanol extracts, respectively. The shells are used as food additives due to their colorant, antioxidant and antimicrobial properties. The shells are exposed by the peeling process applied to obtain the fruit without the shell which is mainly used. The most frequently used technique in chestnut peeling is the Brulage peeling method. However, in this technique, used peeling mechanism is insufficient to obtain both inner and outer shells separately at the same time. Moreover, further research is needed to obtain the shells individually, to analyse each shell in detail, and to increase the industrial use of shells.

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Introduction

Chestnut is the fruit of *Castanea* spp. which belongs to the Fagaceae family. The genus *Castanea* is made up of deciduous trees and shrubs and they primarily grow in the northern hemisphere regions (Nelson et al., 2014). Table 1 displays the ten widely known species of chestnuts. These species are divided in two groups based on the number of nuts in a bur: a) one nut per bur (chinquapins in *Castanea* spp.) and b) three nuts per bur (usually three or more) (Anagnostakis, 2010). The European chestnut (*Castanea sativa* Mill.) originated in the Eastern Mediterranean region during the middle Cretaceous period, and later spread to Europe during the Cenozoic period. Chestnut plantations were begun in the Caucasus Mountains between 900 and 700 BC. It is considered that the name of "Castanea" derives from the city of Kastanea, located near these mountains which was a part of the Pontus Empire. Following the Romans conquest of the Pontus Empire, the cultivation of chestnut throughout Europe (Anagnostakis, 2010; de Vasconcelos et al., 2010a).

According to the Food and Agriculture Organization (2023), among the nuts production worldwide, the chestnut has been ranked number six. I checked the FAO chestnut production data and realised some changes in the data. Gobal chestnut production was 2,134,872 tonnes in 2021, with China contributing approximately 74% (1,570,224 t) of total production. Other significant chestnut producing countries include Spain (187,680 t), Bolivia (83,327 t), Türkiye (77,792 t), the Republic of Korea (52,502t) and Italy (43,000 t). Following these countries, Portugal and Greece recorded production amounts exceeding 30,000 t.

Chestnut fruits have high nutritional value as they contain health-beneficial compounds. The main composition of the dried and peeled European chestnut (*C. sativa*) is reported as carbohydrate (78.43%), water (9.00%), protein (5.01%), fat (3.91%) and ash (3.64%) by the United States Department of Agriculture (2019a). Raw chestnuts can be consumed after boiling or roasting. Additionally, chestnut puree and chestnut candy (marron-glacé) are common chestnut derived products.

Table 1. The classification of *Castanea* spp. based on the number of nuts per bur (Anagnostakis, 2010)

Three nuts per bur		One nut per bur	
Latin name	Common name	Latin name	Common name
Castanea sativa (Miller)	European chestnut	*Castanea pumila* (Miller)	Allegheny chinquapin
Castanea dentata (Marshall) Borkhausen	American chestnut	*Castanea ozarkensis* (Ashe)	Ozark chinquapin
Castanea crenata (Siebold and Zuccarini)	Japanese chestnut	*Castanea floridana* (Sargent) Ashe	Florida chinquapin
Castanea mollissima (Blume)	Chinese chestnut	*Castanea alnifolia* (Nuttal)	Trailing chinquapin
Castanea seguinii (Dode)	Dwarf Chinese chestnut	*Castanea henryi* (Skan) Rehder and Wilson	Chinese chinquapin

Chestnut flour by containing over 50% starch is generally used in pastry after dehydrating and milling of the flesh. Gluten-free and high starchy chestnut flour can be utilised in the production of bread (Demirkesen et al., 2010), gel (Torres et al., 2014), cake ((Yildiz & Dogan, 2014) and chip (Di Monaco et al., 2010), serving as an alternative raw material for celiac patients (Squillaci et al., 2018; Zhu, 2017). Vitamins, minerals, fiber, organic acids, carotenoids and polyphenols (e.g., tannins) are minor components found in chestnuts. Many of these minor compounds possesses antioxidant, anti-carcinogenic, anti-tumor, anti-toxic, anti-inflammatory, anti-microbial and anti-malarial activity (Barreira et al., 2009; de Vasconcelos et al., 2010a; de Vasconcelos et al., 2010b; Goncalves et al., 2010; United States Department of Agriculture, 2019a). Additionally, chestnuts are a good source of dietary fiber due to the presence of indigestible components in the shell (Blaiotta et al., 2013). With these properties, chestnut shell promotes the proliferation of beneficial bacteria, while inhibiting pathogenic bacteria (Xie et al. 2023).

Surrounding the edible part of the chestnut, there are two-layers: a shell coating the flesh and a bur, which are separated as during the peeling process. Other by-products of chestnut processing include leaves, flowers and the tree trunk. Chestnut by-products have various applications in different industries, such as fuel, natural colorant in the food industry, natural antioxidant source in the food, cosmetic, and pharmaceutical industries, as well as in the formulations of wood adhesives and leather tanning (Aires et al., 2016; Cruz-Lopes et al., 2020; Echegaray et al., 2018). The economic value of chestnut by-products is increasing due to their food and non-food applications (Echegaray et al., 2018). Among the various by-products, the shell, which constitutes 10-15% of the chestnut, exhibits the highest antioxidant activity (Gullón et al., 2018; Shen et al., 2023). Rodrigues et al. (2015) analysed the nutritional composition of the *C. sativa* shell from three different production regions in Portugal (Minho, Trás-os-Montes and Beira-Alta). The main components of the shell were carbohydrates (56.51-74.06%), followed by water (21.29-38.61%), protein (2.77-3.13%), ash (1.08-1.60%) and fat (0.15-0.52%). The shell consists of two layers: the inner shell (also known as inner skin, integument, pellicle, seed coat or testa) and the outer shell (also known as outer skin, pericarp, husk, fruit coat or hull). The inner shell is adherent to the fruit flesh, while the outer shell is harder and forms the outermost layer of the fruit (Barreira et al., 2008; de Vasconcelos et al., 2010c; Hwang et al., 2001; Yao et al., 2016; Zamuz et al., 2018).

Industrial Peeling Applications

The economic value of chestnuts is increasing due to their nutritional value and beneficial effects on health. Peeling the chestnuts is necessary before consumption, resulting in a significant amount of inedible chestnut shells being produced. The shell accounts for approximately 10-15% of the chestnut fruit on dry basis (db) (Shen et al., 2023). Assuming the FAO data on the chestnut production in the world, over 230,000 tonnes (db) of chestnut shells are expected to emerge from the processes. These shells are of great significance as they contain high levels of antioxidant polyphenols, and pigments, minerals, vitamins, dietary fiber, essential fatty acids, and essential amino acids (Squillaci et al., 2018; United States Department of Agriculture, 2019a; Yao & Qi, 2016). These by-products can be utilized as fuel in factories and in various industries such as leather tanning, bioenergy, painting, cosmetic, nutraceutical and pharmaceutical industries. Additionally, in the food industry, these by-products are used as food additives due to their colorant, antioxidant and antimicrobial properties (Aires et al., 2016; Chen et al., 2018; Echegaray et al., 2018; Shen et al., 2023; Zhu et al., 2022). Moreover, in a recent study, it was stated that the inner and outer shells of chestnuts have a high potential in obtaining the products required for bioenergy production by pyrolysis, and even the pyrolysis products of the inner shell consist of more gaseous products (Shen et al., 2023). Since the two-layered shell has different compositions and distinct areas of use, it is important to separate shells from the flesh, and from each other. de Vasconcelos et al. (2010c) reported that four Portuguese chestnut cultivars (*C. sativa*) produce inner shells ranging from 6.33-10.10% and outer shell ranging from 8.96-13.54% of the fresh weight of the whole fruit.

Since there is a stronger interest in the inner shell, it becomes important to obtain the shells individually. It should be noted that separating the flesh from the inner and outer shells is challenging due to their adhesiveness. Tanaka et al. (1981) reported that tannins were relatively more abundant in the inner shell and reacted with proteins or polysaccharides to form complexes. These complexes may contribute to the adhesion of the inner shell. The findings of Hara et al. (1995) supported this inference, as they discovered that tannin accumulation in the inner shell led to the bonding of the shell to the flesh. The results of the study conducted by Hwang et al. (2001) are consistent with these findings. They analysed 14 chestnut varieties (*C. crenata*) and found a significant negative correlation

between the tannin content of the inner shell and the peeling ratio, as well as a positive relationship between the tannin content of the outer shell and the peeling ratio.

The initial peeling process of the shells was done by hand. However, to reduce labour requirements and shorten processing time, researchers began searching for new peeling techniques that combine physical and chemical treatments (Kim et al., 1997; Oh et al., 1985). Hwang et al. (2001) developed a machine that integrate high temperature and mechanical scraping to peel off both shells, but it was not sufficient for practical applications. Industrially, the most known and preferred peeling method worldwide is called Brulage peeling (Figure 1). This process results in two types of shell residues: a blend of inner and outer shells, and the inner shell itself. The Brulage peeling process consists of four parts. Firstly, chestnuts with the shell intact are fed into a burner. They are then conveyed through an oven using a screw auger cage to make the peel brittle. Next, they are passed into a trasher containing rubber-ended paddles that move against steel rods, breaking away the outer shell and, in some cases, parts of the inner shell. The chestnuts are then conveyed to a steamer, which is a closed screw conveyor partially filled with water and heated with steam to 70-80°C (158-176°F) to loosen any remaining inner shell. Finally, the chestnuts are moved onto a brusher/washer, where loose inner shell is removed using counter-rotating pairs of rollers, followed by a cleaning rinse (Squillaci et al., 2018; Yen, 2006).

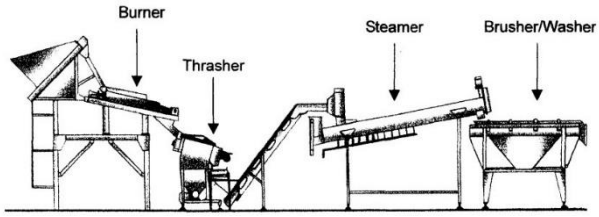

Figure 1. Schematic of process steps of the Brulage peeling line (Yen, 2006)

Bioactive Compounds

Antioxidant Polyphenols

There have been several studies demonstrating that chestnut (*C. sativa*) fruit and by-products contain a considerable amount of phenolic compounds (Barreira et al., 2008; Vella et al., 2018; Živković et al., 2009). Barreira et al. (2008) investigated the total phenolic content (TPC) of different parts of the chestnut plant and reported that the shells had the highest TPC. Conversely, Vella et al. (2018) and Živković et al. (2009) determined the highest TPCs for leaves (90.35 mg GAE/g db) and flowers (33 mg GAE/g dry extract (de)), respectively. Vazquez et al. (2008) stated that TPC of chestnut *(C. sativa)* shell extracts ranged between 26.6 and 59.7 g GAE/100 g extract. The total phenolic content of chestnut shells reported by different authors is presented in Table 2.

The phenolic extract of the shells consists of different groups of compounds such as *ortho*-diphenols, flavonoids and tannins (Squillaci et al., 2018). The number of included active groups (OH or NH_2) and their positions affect the antioxidant capacity of phenolics. The *ortho*- position is more active than *para*- and *meta*- positions of compounds. The antioxidant properties of *o*-diphenols can be attributed to hydrogen donation, i.e., their ability to improve radical stability by forming an intramolecular hydrogen bond between free hydrogens of their hydroxyl group and their phenoxyl radicals (Bendary et al., 2013; Visioli & Galli, 1998). Squillaci et al. (2018) analysed the *o*-diphenols contents of the inner and a combination of the inner and outer shells of chestnut (*C. sativa*) (Table 2). The amounts of *o*-diphenols of the inner and the combined shells were 19.55 and 98.06 mg CAE/g de, respectively. As can be understood from this result, when compared with the inner shell, outer one has much more *o*-diphenols content.

Additionally, it was observed that there was a direct relationship between the TPC and antioxidant activity (AA) of chestnut shells (Vazquez et al., 2008; Vella et al., 2018). In a study carried out conducted by Barreira et al. (2008), the AA of different parts of chestnut (leaf, flower, fruit, inner and outer shells) were analysed using five different biochemical assays including DPPH radicals scavenging activity, reducing power, inhibition of β-carotene bleaching, hemolysis inhibition and inhibition of lipid peroxidation. Among all assays, chestnut shells, especially the outer shell, exhibited the highest AA. The results of other studies as regards antioxidant activity of shells are given in Table 3.

Seo et al. (2016) conducted an investigation on the DNA protection and antioxidant potential of chestnut (*C. crenata*) shell extracts. They determined the radical-scavenging activity using an Electron Spin Resonance spectrometer and evaluated the protection against oxidative DNA damage. The assays applied included DPPH, ABTS, nitrite, hydroxyl, superoxide, reducing power, inhibition of linoleic acid oxidation, and prevention of oxidative DNA damage (Table 3). The extracts showed significantly antioxidant activity.

Sorice et al. (2016) conducted research to determine the potential anti-cancer effects of polyphenols extracted from chestnut (*C. sativa*) shells testing them on six different human cell lines (A375, H460, HT29, MCF7, HepG2, and HaCaT). After 48 h of treatment with extracted polyphenols, only HepG2 cells showed inhibition relative to EC_{50}, along with increased apoptosis and mitochondrial depolarization. The evaluation of the cytokinome before and after treatment revealed a decrease in vascular endothelial growth factor and the tumor necrosis factor, suggesting potential anti-angiogenic and anti-inflammatory effects of the extract. In conclusion, it was observed that polyphenols had significant effects on biomolecules related to cell proliferation, apoptosis, cell cycle and mitochondrial depolarization, as well as on cytokinomes and metabolomics profiles. It was also considered that bioactive compounds of chestnut shell were very resistance to *in vitro* digestion conditions. With these properties, chestnut shells were evaluated as a promising ingredient for the delivery of polyphenols in nutraceutical studies (Pinto et al. 2023).

Noh et al. (2010a) demonstrated the antioxidant effects of chestnut (*C. crenata*) inner shell extract in cell line and oxidative stress-induced animal models. The study showed that inner shell stimulation increased antioxidant enzyme activities while simultaneously decreasing lipid peroxidation in *tert*-butylhydroperoxide-treated HepG2 cells.

Table 2. Polyphenol content of chestnut shells

Latin name	Outer shell					References
	TP	*o*-d	F	T	CT	
C. sativa	4.18-5.95 mg GAE/g db	2.68-3.71mg CAE/g db	2.34-2.80mg CE/g db	0.70-1.44mg GAE/g db		(Vella et al., 2019)
C. sativa	510mg GAE/g extract		503mg CE/g extract			(Barreira et al., 2008)
C. sativa	12mg GAE/g de		6.5mg CE/g de			(Zivkovic et al., 2009)
C. sativa	2.22-105.66mg GAE/g wb					(de Vasconcelos et al., 2010c)
C. sativa	45.01mg GAE/g db					(Mustafa et al., 2021)
C. crenata					0.31-2.04% (db)	(Hwang et al., 2001)
C. sativa × *C. crenata*	3.62mg GAE/g db	2.32mg CAE/g db	1.74mg CE/g db	1.11mg GAE/g db		(Vella et al., 2019)
Latin name	Inner shell					References
	TP	*o*-d	F	T	CT	HT
C. sativa	212.82-251.76mg GAE/g db	101.99-116.17mg CAE/g db	82.84-103.15mg CE/g db	31.00-36.46mg GAE/g db			(Vella et al., 2019)
C. sativa	475mg GAE/g extract		330mg CE/g extract				(Barreira et al., 2008)
C. sativa	3.37-136.35mg GAE/g wb						(de Vasconcelos et al., 2010c)
C. sativa	54.04mg GAE/g db						(Mustafa et al., 2021)
C. crenata					7.83-71.42% (db)		(Hwang et al., 2001)
C. sativa × *C. crenata*	337.33mg GAE/g db	191.47mg CAE/g db	129.14mg CE/g db	107.91mg GAE/g db			(Vella et al., 2019)
C. sativa	43.69mg GAE/g de	19.55mg CAE/g de	7.94mg CE/g de		25.84mg GAE/g de	2.02mg GAE/g de	(Squillaci et al., 2018)
C. crenata	264.10-558.12mg GAE/g extract		47.41-166.28mg CE/g extract		85.13-244.63mg CE/g extract		(Ham et al., 2015)
Latin name	Shell					References
	TP	*o*-d	F	T	CT	HT
C. sativa	205.99mg GAE/g de	98.06mg CAE/g de	40.98mg CE/g de		162.49mg GAE/g de	12.94mg GAE/g de	(Squillaci et al., 2018)
C. sativa	190.12-312.44mg GAE/g de	73.90-148.72mg CAE/g de	47.75-62.18mg CE/g de	118.97-205.99mg GAE/g de			(Cacciola et al., 2019)
C. sativa	2.38-17.68mg GAE/g db	1.11-8.29mg CAE/g db	7.36mg CE/g db	3.48mg GAE/g db			(Vella et al., 2018)
C. sativa	533.81-805.74mg GAE/g extract		49.92-146.08mg CE/g extract				(Barreira et al., 2010)
C. sativa	143.00-796.80mg GAE/g db		31.38-43.33mg CE/g db				(Rodrigues et al., 2015)
C. sativa	33.32-49.14g GAE/100 g extract						(Nazzaro et al., 2012)
C. sativa	590.2g GAE/kg de						(Sorice et al., 2016)
C. crenata	136.12-353.92mg GAE/100 mg db		367.43-459.09mg CE/100 mg db				(Seo et al., 2016)

TP, Total Phenols; *o*-d, *ortho*-diphenols; F, Flavonoids; T, Tannins; CT, Condensed Tannins, HT, Hydrolysable Tannins; GAE, Gallic Acid Equivalents; CAE, Caffeic Acid Equivalents; CE, Catechin Equivalents; db, dry basis; wb, wet basis; de, dry extract

Table 3. Antioxidant activity of shells

Latin name	AB	DPPH radical	RP	β	FRAP	Unit	References
				Outer shell			
C. sativa		39.7	55.1	133		μg/ml (EC_{50})	(Barreira et al., 2008)
C. sativa		27.1-79.2(μg/ml (EC_{50}))			2.25-5.11(mg AAE/g db)		(Vella et al., 2019)
C. sativa		21.4				% (0.2 mg extract/ml solution)	(Zivkovic et al., 2009)
C. sativa		5.04				mg TE/g db	(Mustafa et al., 2021)
C. sativa × *C. crenata*		77.9(μg/ml (EC_{50}))			1.82(mg AAE/g db)		(Vella et al., 2019)
				Inner shell			
C. sativa		32.7	68.7	164		μg/ml (EC_{50})	(Barreira et al., 2008)
C. sativa		32.9-35.3(μg/ml (EC_{50}))			113.63-149.27(mg AAE/g db)		(Vella et al., 2019)
C. sativa		5.25				mg TE/g db	(Mustafa et al., 2021)
C. crenata	270.73	23.81	187.78	93.03 (LPI %)		μg/ml (EC_{50}) free phenolic extract	(Tuyen et al., 2017)
C. crenata	538.70	28.41	209.56	93.35 (LPI %)		μg/ml (EC_{50}) bound phenolic extract	(Tuyen et al., 2017)
C. crenata		159.79-174.61				mM TE/g extract	(Ham et al., 2015)
C. sativa × *C. crenata*		35.8(μg/ml (EC_{50}))			113.25(mg AAE/g db)		(Vella et al., 2019)
				Shell			
C. sativa					475-3808	nmol AAE/mg extract	(Vazquez et al., 2008)
C. sativa					624-3555	nmol AAE/mg extract	(Vazquez et al., 2009)
C. sativa					21.65-105.62	mmol AAE/ 100 g db	(Leclercq et al., 2010)
C. sativa					2268-3779	nmol AAE/mg extract	(Nazzaro et al., 2012)
C. sativa					13.62	mg AAE/g db	(Vella et al., 2018)
C. sativa		78.5				% inhibition	(Sorice et al., 2016)
C. sativa		82.41-159.99	79.25-117.58	74.62-151.27		μg/ml (EC_{50})	(Barreira et al., 2010)
C. sativa		31.80-37.61 μg/ml (EC_{50})			6008.70-8083.50μmol ferrous sulphate/g db		(Rodrigues et al., 2015)
C. crenata	98.60-99.59	92.85-96.13				% (2 mg extract/ml solution)	(Seo et al., 2016)
Latin name	HI	Hydroxyl radical	TBARS	NR	Unit		References
				Outer shell			
C. sativa	91.4		7.87		μg/ml (EC_{50})		(Barreira et al., 2008)
C. sativa							(Vella et al., 2019)
C. sativa		21.8			% (0.2 mg extract/ml solution)		(Zivkovic et al., 2009)
C. sativa					mg TE/g db		(Mustafa et al., 2021)
				Inner shell			
C. sativa	47.5		11.5		μg/ml (EC_{50})		(Barreira et al., 2008)
C. sativa							(Vella et al., 2019)
				Shell			
C. sativa			27.29-49.07		μg/ml (EC_{50})		(Barreira et al., 2010)
C. crenata				28.55-32.85	% (2 mg extract/ml solution)		(Seo et al., 2016)

AB: ABTS radical; HI: Hemolysis inhibition; RP: Reducing power; TBARS: TBARS inhibition (lipid peroxidation); β: β-carotene bleaching inhibition; NR: Nitrite radical; LPI, Lipid Peroxidation Inhibition; TE, Trolox Equivalent; AAE, Ascorbic Acid Equivalent; db, dry basis

The inner shell extract also inhibited lipid peroxidation in the livers of mice fed a high-fat diet and treated with CCl_4. The researchers attributed this finding to scoparone and scopoletin. In addition, in an oxidative stress-induced in in vitro system, the antioxidant capacity of scopoletin was reported to be relatively higher than that of scoparone. With a such high antioxidant capacity of chestnut shell, it is suggested as an effective antioxidant for future *in vivo* research and drug application (Hu et al. 2021).

Phenolic Acids and Flavonoids

Two subgroups of phenolic compounds are phenolic acids and flavonoids. Phenolic acids consist of hydroxycinnamic acids with a phenylpropane (C_6-C_3) structure, and hydroxybenzoic acids with a phenylmethane (C_6-C_1) structure (Acar & Gökmen, 2014). Flavonoids, on the other hand, are composed of flavan-3-ols, flavonols, flavones, isoflavones, flavanones and anthocyanidins, with their composition depending on the oxidation state of the central C ring (Dai & Mumper, 2010; Gan et al., 2019). These compounds are present not only in chestnuts but also in other fruits and vegetables. Barreira et al. (2008) reported that the by-product parts of *C. sativa* contain a significant amount of flavonoids. They noted that the outer shells of chestnuts (503 mg CE/g) had a higher flavonoid content than the inner ones (330 mg CE/g). Similar findings were reported by Squillaci et al. (2018) (Table 2). In another study, which reported lower values than the aforementioned research, Ham et al. (2015) examined the inner shell of chestnut (*C. crenata*) and determined its total flavonoid content to be 47.41-166.28 mg CE/g. The differences in results could be attributed to species diversity, ecological factors and extraction conditions. Liu et al. (2020) found that rutin and quercetin were the main flavonoids in chestnut shell.

In various studies conducted to determine phenolics profile of the chestnut shells (Table 4), gallic acid was reported as the most abundant phenolic compound (Nazzaro et al., 2012; Sorice et al., 2016; Squillaci et al., 2018; Rodrigues et al., 2023). Moreover, considerable amounts of protocatechuic and ellagic acids were also reported. Minor phenolic acids of chestnut shells were chlorogenic, syringic, *p*-coumaric and ferulic acids. Only Sorice et al. (2016) determined syringic acid in the shell of *C. sativa*, and provided an approximate result due to interference from condensed tannins. The total content of phenolic acids (12 phenolic acids) was reported as 223.62 and 119.17 mg/kg db for the inner and outer shells of *C. sativa*, respectively (Mustafa et al., 2021).

Catechins and their derivatives, involved in the flavan-3-ols group of flavonoids, are classified based on their structures as catechin, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate. Among the identified catechin derivatives in the chestnut shells so far are catechin, epicatechin and epigallocatechin (Table 4). Nazzaro et al. (2012), Aires et al. (2016) and Squillaci et al. (2018) detected catechin, epicatechin and epigallocatechin in the chestnut (*C. sativa*) shells by HPLC (RP-HPLC, HPLC-DAD/VIS-MS, HPLC/UV, respectively). Epigallocatechin was also reported in the shells by Aires et al. (2016). Additionally, Sorice et al. (2016) qualitatively determined the presence of epicatechin and epigallocatechin in the shell extracts. Squillaci et al. (2018) observed that the inner shell and the combined inner and outer shells contained catechin in amount of 0.30 and 0.70 mg/g de, respectively, and epicatechin in amount of 0.32 and 0.71 mg/g de, respectively. The results showed that the contents of catechin derivatives in the outer shell were higher than those in the inner shell. The study of Mustafa et al. (2021) declared the differences in the amount of catechin and epicatechin between the inner and outer shells. In addition, for both shells, while dihydrocalcones (phloridzin and phloretin) were detected, flavanones (hesperidin and naringin) were not found in either shell. Despite their small amounts, rutin and quercetin from flavonols were identified in the chestnut shells (Nazzaro et al., 2012; Sorice et al., 2016). Along with these compounds, quercitrin, isoquercitrin, hyperoside, myricetin, isorhamnetin, kaempferol and kaempferol-3-glucoside from other flavonol compounds were also observed in both shell structures (Mustafa et al., 2021). It should also be noted that the content of these compounds varied with species diversity, origin, ecological factors, storage and selected extraction conditions (Cerulli et al. 2020).

Tannins

Characteristically, tannins have a tendency to bind and precipitate proteins. The ability of tannins to remove gelatin and other proteins in raw juices, as well as collagen proteins in animal hides, plays a crucial role in the juice and tanning industries, respectively. Tannins also cause sensations of astringency, which is characterized by feeling of dryness and puckering in the mouth. This occurs when tannins bind to salivary proteins (Crozier et al., 2006; Lee & Lawless, 1991; McRae & Kennedy, 2011). Tannins show various beneficial aspects, including antioxidant, anti-cancer, antimicrobial, anti-nutritional, anti-diabetic, anti-obesity and cardio-protective effects.

Tannins can be classified into two groups based on their structure: hydrolysable and condensed tannins. Hydrolysable tannins can be further divided into gallotannins, which are esters of glucose (most often β-D-glucose) or another polyol with gallic acid) and ellagitannins (with hexahydroxydiphenic acid) (Mammela et al., 2000; Salminen et al., 1999). The term "ellagitannin" is derived from ellagic acid, which is formed spontaneously from hexahydroxydiphenic acid in an aqueous solution through an intra-molecular esterification reaction (Vermerris & Nicholson, 2006). The main ellagitannins identified in chestnut include vescalagin, castalagin, vescalin, castalin, chestanin, acutissimin A and acutissimin B (de Vasconcelos et al., 2010c; Esposito et al., 2019; Mannelli et al., 2019; Martinez & Stagljar, 2003). Additionally, condensed tannins are oligomers and polymers of flavan-3-ol (catechin and its derivatives) units. They are referred to as catechin tannins, procyanidins, and proanthocyanidins (Anonymous, 2003; Karonen et al., 2004).

Tannin content of chestnut shell was reported as higher than that of phenolic acids and flavonoids (Pinto et al. 2021). Vella et al. (2018) reported the tannin content of chestnut shells as 3.48 mg GAE/g db. Rodrigues et al. (2023) determined total hydrolysable and condensed tannin content of chestnut shell as ranged between 1.00-3.33 and 1.02-3.58 mg/g, respectively.

Table 4. Phenolic acids, flavonoids and tannins profile of chestnut (*C. sativa*) shells

Phenolic acids							Unit	References
Gal	Pro	Ell	Chl	Syr	Cou	Fer		
Outer shell								
69.20		35.49	0.06	0.79	0.25	2.37	mg/kg db	(Mustafa et al., 2021)
0.14-0.36		0.14-0.19					mg/g wb	(de Vasconcelos et al., 2010c)
0.60-1.08		0.24-0.90					mg/g db	(Vella et al., 2019)
		0.74-5.98					mg/g db	(Vekiari et al., 2008)
Inner shell								
118.85		86.35	0.07	0.96	1.27	4.82	mg/kg db	(Mustafa et al., 2021)
29.62	3.43	0.63			0.32		mg/g de	(Squillaci et al., 2018)
0.22-0.35		0.03-0.07					mg/g wb	(de Vasconcelos et al., 2010c)
3.37-6.67		0.80-1.38					mg/g db	(Vella et al., 2019)
		0.54-0.79					mg/g db	(Vekiari et al., 2008)
Shell								
86.97-150.09	11.20-21.57	0.58-1.09	0.67-1.18	0.14-0.21	0.22-0.52	0.03-0.31	mg/g de	(Cacciola et al., 2019)
63.51	11.24	0.81			0.22		mg/g de	(Squillaci et al., 2018)
7.9-584.9		47.6-3542.6					µg/g db	(Aires et al., 2016)
4.53-9.31		0.04-1.05	0.02-0.54		0.16-0.23	0.01-0.20	µg/mg extract	(Nazzaro et al., 2012)
2.12		1.05		0.50			g/kg db	(Sorice et al., 2016)
Flavonoids							Unit	References
Cat	Epic	Epig		Rut	Que			
Outer shell								
91.34	7.94			2.45	0.79		mg/kg db	(Mustafa et al., 2021)
					n.d.		mg/g db	(Vella et al., 2019)
Inner shell								
110.93	6.30			3.48	0.53		mg/kg db	(Mustafa et al., 2021)
0.30	0.32						mg/g de	(Squillaci et al., 2018)
					n.d.		mg/g db	(Vella et al., 2019)
Shell								
	0.71-1.28						mg/g de	(Cacciola et al., 2019)
0.70	0.71						mg/g de	(Squillaci et al., 2018)
15.1-295.9	9.0-91.6	13.6-213.4					µg/g db	(Aires et al., 2016)
0.38-1.33	0.07-0.95			0.05			µg/mg extract	(Nazzaro et al., 2012)
				0.059	0.081		g/kg db	(Sorice et al., 2016)
Tannins					Hydroxy-coumarins		Unit	References
Ves	Cas	AcuA	AcuB		Sco			
Outer shell								
0.05-0.13	0.39-0.85	0.05-0.08	0.41-0.52				mg/g wb	(de Vasconcelos et al., 2010c)
Inner shell								
					0.41		mg/g de	(Squillaci et al., 2018)
0.04-0.08	0.07-0.21	0.03-0.05	0.04-0.09				mg/g wb	(de Vasconcelos et al., 2010c)
Shell								
					0.11-0.41		mg/g de	(Cacciola et al., 2019)
					0.11		mg/g de	(Squillaci et al., 2018)
67.5-109.4	49.6-100.4	n.d.	n.d.				µg/g db	(Aires et al., 2016)

(Gal, Gallic acid; Pro, Protocatechuic acid; Ell, Ellagic acid; Chl, Chlorogenic acid; Syr, Syringic acid; Cou, *p*-Coumaric acid; Fer, Ferulic acid; Cat, Catechin; Epic, Epicatechin; Epig, Epigallocatechin; Rut, Rutin; Que, Quercetin; Ves, Vescalagin; Cas, Castalagin; AcuA, Acutissimin A; AcuB, Acutissimin B; Sco, Scopoletin; n.d., not detected; wb, wet basis; db, dry basis; de, dry extract)

The contents of condensed and hydrolysable tannins related to the chestnut shell structure were provided in Table 4. In relation to these contents, Squillaci et al. (2018) conducted a study on the inner and combined shells of *C. sativa*. The amount of condensed tannins in both the inner shell (25.84 mg GAE/g de) and shell combination (162.49 mg GAE/g de) was higher than that of hydrolysable tannins (2.02 mg GAE/g de and 12.94 mg GAE/g de, respectively). Hence, the outer shells had higher contents of both condensed and hydrolysable tannins compared to the inner shells. However, in *C. crenata* chestnut variety, Hwang et al. (2001) determined that the condensed tannins content of the inner shell was higher than that of the outer shell. The total procyanidins (condensed tannins) of the inner and outer shells of chestnut were reported as 6.28-110.35 and 3.14-35.11 mg/g wb by de Vasconcelos et al. (2010c). Ellagitannins, including vescalagin, castalagin, acutissimin A and acutissimin B were analysed in the chestnut shells (Aires et al., 2016; de Vasconcelos et al., 2010c). All these compounds were found in both shells according to de Vasconcelos et al. (2010c), but; in the study by Aires et al. (2016), acutissimin A and acutissimin B were not detected.

Table 5. Minor compounds of unpeeled and peeled European chestnut (*C. sativa*) on wet and dry bases (United States Department of Agriculture, 2019a, 2019b, 2019c, 2019d)

	Unity	Wet Basis			Dry Basis		
	(per 100 g)	unpeeled	peeled	shell	unpeeled	peeled	shell
Minerals							
Ca	mg	27	19	8	67	64	3
Fe	mg	1.01	0.94	0.07	2.38	2.39	
Mg	mg	32	30	2	74	74	0
P	mg	93	38	57	175	137	38
K	mg	518	484	34	986	991	
Na	mg	3	2	1	37	37	0
Zn	mg	0.52	0.49	0.03	0.35	0.35	0
Cu	mg	0.447	0.418	0.029	0.65	0.653	
Mn	mg	0.952	0.336	0.616	1.3	1.183	0.117
Vitamins							
Vitamin C, total ascorbic acid	mg	43	40.2	2.8	15	15.1	
Thiamin	mg	0.238	0.144	0.094	0.295	0.354	
Riboflavin	mg	0.168	0.016	0.152	0.36	0.054	0.306
Niacin	mg	1.179	1.102	0.077	0.85	0.854	
Panthotenic acid	mg	0.509	0.476	0.033	0.897	0.901	
Vitamin B-6	mg	0.376	0.352	0.024	0.663	0.666	
Folate, total	µg	62	58	4	109	110	
Folic acid	µg	0	0	0	0	0	0
Folate, food	µg	62	58	4	109	110	
Folate, DFE	µg	62	58	4	109	110	
Vitamin B-12	µg	0	0	0	0	0	0
Vitamin A, RAE	µg	1	1	0	0	0	0
Retinol	µg	0	0	0	0	0	0
Vitamin A, IU	IU	28	26	2	0	0	0
Vitamin D (D2+D3)	µg	0	0	0	0	0	0
Vitamin D	IU	0	0	0	0	0	0
Fatty acids, total saturated	g	0.425	0.235	0.190	0.837	0.736	0.101
14:0	g	0.01	0.005	0.005	0.019	0.017	0.002
16:0	g	0.384	0.212	0.172	0.755	0.664	0.091
18:0	g	0.021	0.012	0.009	0.042	0.037	0.005
Fatty acids, total monounsaturated	g	0.78	0.43	0.350	1.535	1.349	0.186
16:1	g	0.021	0.012	0.009	0.042	0.037	0.005
18:1	g	0.749	0.413	0.336	1.473	1.296	0.177
20:1	g	0.01	0.005	0.005	0.019	0.017	0.002
Fatty acids, total polyunsaturated	g	0.894	0.493	0.401	1.758	1.546	0.212
18:2	g	0.798	0.44	0.358	1.57	1.381	0.189
18:3	g	0.095	0.053	0.042	0.188	0.165	0.023
Aminoacids, essential							
Arginine	g	0.173	0.116	0.057	0.457	0.359	0.098
Histidine	g	0.067	0.045	0.022	0.177	0.139	0.038
Threonine	g	0.086	0.058	0.028	0.228	0.179	0.049
Isoleucine	g	0.095	0.064	0.031	0.252	0.198	0.054
Leucine	g	0.143	0.096	0.047	0.378	0.297	0.081
Lysine	g	0.143	0.096	0.047	0.378	0.297	0.081
Methionine	g	0.057	0.038	0.019	0.151	0.118	0.033
Phenylalanine	g	0.102	0.069	0.033	0.27	0.212	0.058
Valine	g	0.135	0.091	0.044	0.357	0.280	0.077
Tryptophan	g	0.027	0.018	0.009	0.071	0.056	0.015
Amino acids, non-essential							
Cystine	g	0.077	0.052	0.025	0.202	0.159	0.043
Alanine	g	0.161	0.109	0.052	0.427	0.335	0.092
Tyrosine	g	0.067	0.045	0.022	0.177	0.139	0.038
Aspartic acid	g	0.417	0.281	0.136	1.103	0.886	0.217
Glutamic acid	g	0.312	0.21	0.102	0.824	0.647	0.177
Glycine	g	0.124	0.084	0.040	0.329	0.258	0.071
Proline	g	0.127	0.086	0.041	0.336	0.264	0.072
Serine	g	0.121	0.081	0.040	0.319	0.251	0.068

(The values in the column for shells, not included in the original data, were obtained by subtracting the values of peeled chestnuts from those of unpeeled one.)

Hydroxycoumarins

Hydroxycoumarins are a group of phenolic compounds similar to phenolic acids, flavonoids, tannins, etc. Scopoletin and scoparone, which are coumarins, have been found in chestnut shells by various researchers (Jung et al., 2019; Noh et al., 2010a; Noh et al., 2011; Noh et al., 2010b; Squillaci et al., 2018). In the study by Squillaci et al. (2018), it was determined that scopoletin content was 0.41 mg/g de in the inner shell and 0.11 mg/g de in the combined shells of *C. sativa*. As the result suggests, inner shell is the main source of scopoletin for chestnut. From the inner shell extract of *C. crenata*, Noh et al. (2010a) and Noh et al. (2011) isolated two compounds, scoparone and scopoletin, as the main components using repeated column chromatography. Noh et al. (2010a) investigated the effect of scoparone and scopoletin on *t*-BHP treated HepG2 cells under oxidative stress conditions, focusing on intracellular ROS generation and antioxidant enzyme activities. They confirmed that both compounds have antioxidant effects, with scopoletin demonstrating relatively higher antioxidant capacity than scoparone in an in vitro oxidative stress-induced system. The findings of Noh et al. (2011) suggest that scoparone provides a stronger antioxidant effect than scopoletin under oxidative stress induced by ethanol in an in vitro system. These studies revealed that the inner shell extract of chestnut has an inhibitory effect on lipid accumulation and a preservative effect on antioxidant potential in the liver. Furthermore, chestnut inner shell showed protective effects against ethanol-induced oxidative damage, potentially due to its inhibition of lipid accumulation, peroxidation and the enhancement of the antioxidant defense system in the liver. Scopoletin, scoparone and quercetin have been referred to as "natural pesticide" by Sanzani et al. (2014) due to their antifungal characteristics and antioxidant properties.

Pigments

Chestnut shells, which are by-products of chestnut processing, contain natural brown pigments. Due to their strong coloring power, antioxidant activity and bacteriostatic effects, they can be evaluated as a food additive (Chen et al., 2018; Gao et al., 2019; Li & Song, 2004; Yao & Qi, 2016). The pigments in chestnut shells associated with lignin and cellulose in cell wall components (Zhu et al., 2022). Chestnut shells primarily consist of Klason lignin, α-cellulose and hemicellulose structures. The high lignin content makes them suitable for use in the adhesive sector (Cruz-Lopes et al., 2020; Gullón et al., 2018). Furthermore, positive findings have been observed in hair coloring due to colorant properties of chestnut shells (Rose et al., 2018; Zhu et al., 2022). These natural pigments have been reported to contain 15% melanin (Yao & Qi, 2016; Yao et al., 2016; Yao et al., 2012). Melanin has also isolated from various food sources as *Osmanthus fragrans'* seeds (Wang et al., 2006), black cumin (*Nigella sativa* L.) seeds (Al-Mufarrej et al., 2006), silky fowl (Chen et al., 2008) and Atlantic salmon (Leclercq et al., 2010). Melanins produced by bacteria, fungi, animals and plants, and their structures are enigmatic, complex and variable. It is worth noting that melanins are large, extended, amorphous, irregular, heterogeneous, highly crosslinked and hydrophilic (Enochs et al., 1993; Łopusiewicz, 2016).

Melanins are categorized into three groups according to the type of polymerization: eumelanins, pheomelanins and allomelanins. Eumelanins are generated by polymerization of a nitrogenous melanogen. Pheomelanins are generated by the polymerization of a sulphurated melanogen. Additionally, allomelanins are also generated by polyphenols (Velisek et al., 2007). Allomelanins, which are a heterogeneous group and commonly found in some fungi, higher plants. These are pigments that show color range from dark brown to black comprising polymers of simple phenols and their quinones (Hendry, 1995).

Yao and Qi (2016) reported a relationship between melanin and antioxidant activity, citing the investigations of Sava et al. (2001) and Huang et al. (2011). The antioxidant activity of melanins depends on their oxidative state and chemical structures. The oxidation of melanins decreases the antioxidant capacity, leading to the formation of quinone groups from phenolic ones. Consequently, the phenolic content serves as an indicator to evaluate the oxidative degree of melanins (Hung et al., 2002; Yao & Qi, 2016). Additionally, melanins have been reported to have other beneficial effects, including immune-stimulating properties (Sava et al., 2001), anti-HIV (Montefiori & Zhou, 1991), anti-venin (Hung et al., 2004), anti-tumor (Kamei et al., 1997) and radioprotection (Hill et al., 1987) activities.

Other Bioactive Phytochemicals

In Table 5 (United States Department of Agriculture, 2019a, 2019b, 2019c, 2019d), minerals, vitamins, essential fatty acids and essential amino acids contents of unpeeled, peeled and shell of fresh and dried European chestnuts (*C. sativa*) are provided. The difference in values between unpeeled and peeled chestnuts provides information about the properties of the shell. Some minerals such as phosphorus (38 mg/100 g), calcium (3 mg) and manganese (0.117 mg), as well as some vitamins like riboflavin (0.306 mg), can be found in the shell structure. The presence of vitamin C in chestnut (*C. crenata*) shell extracts was determined by Seo et al. (2016) who determined vitamin C levels to be 15.57 and 28.97 mg AA/100 mg db in water and ethanol extracts, respectively. Rodrigues et al. (2015) examined the shell of three European chestnut varieties for total vitamin E content and found that the total vitamin E content of the shells ranged from 481 to 962 mg/100 g sample. The author also declared that γ-tocopherol was the precursor of E vitamin in shells. On the other hand, de Vasconcelos et al. (2010c) analysed α-, γ- and δ-tocopherols in the inner and the outer shells of *C. sativa*, but did not detect any of them. The differences between studies could be related to the growing conditions and ecological practices.

Table 5 indicates that the quantities of total fatty acids in the shells of fresh and dried European chestnuts are 0.941% and 0.499%, respectively. The rate of essential fatty acids (linoleic and linolenic acids) in the shells is 42.5% for each sample.

Chestnut shell was found to be a rich source of amino acid. Rodrigues et al. (2015) also investigated the amino acid content of chestnut shells, and detected 17 different amino acids, including non-essential (alanine, serine, aspartic acid, glutamic acid, glycine, proline, tyrosine and ornithine) and essential (histidine, isoleucine, leucin, lysine, phenylalanine, threonine, arginine, valine and

methionine). Among these amino acids, arginine was the dominant one, with levels ranging from 355 to 721 mg/100 g db, while ornithine had the lowest concentration in the shell ranging from 1 to 9 mg/100g db.

Conclusion

This review summarizes the peeling applications of chestnut (*Castanea* spp.) and highlights the bioactive compounds of shells. Evaluating these shells is important from both environmental and health perspectives. These by-products have significant economic and nutritional value due to their high content of bioactive compounds such as phenolic acids, flavonoids, tannins, hydroxycoumarins, pigments and certain vitamins. Over time, the unknown properties of the inner and outer shells have emerged, and the assessment of both shells can differ. Therefore, there is a need for peeling machines that minimise loss and maximise efficiency, allowing to the separate collections of shells.

In addition to their anti-wrinkle, anti-aging, antioxidant, antibacterial and anticancer effects, the inner shell of the chestnut gains more importance in the pharmaceutical industry due to its anti-asthmatic properties. Moreover, it has gained attention in the cosmetic industry for its high deodorizing effect and potential as a natural source for hair dyeing. Chestnut shells can also be utilised as colorant, antioxidant and antimicrobial agents in the food, food supplement and nutraceutical industries.

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Conflict of interest

The author declares no conflict of interest.

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