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The Effects of Different Doses of Zeatin, Kinetin and Gibberellic Acid Biostimulants Applied during the Seedling Development Period of Peppermint (*Mentha Piperita* L.) on Growth and Biochemical Parameters

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A B S T R A C T

This study aimed to evaluate the impact of varying doses of Zeatin, Kinetin, and Gibberellic Acid biostimulants on the growth and biochemical parameters of *Mentha piperita* L. Conducted in a greenhouse with three replications using a "Completely Randomized Experimental Design" design, the experiment assessed seedling and root lengths, fresh and dry weights of seedlings and roots, total phenolic content, and antioxidant activity (CUPRAC and FRAP). The results revealed that biostimulant applications significantly increased all growth and biochemical parameters compared to the control. Gibberellic acid at 200 mg/l produced the longest seedlings, while Kinetin at 50 mg/l resulted in the longest roots. The highest antioxidant activity (FRAP) and total phenolic content were observed with the 40 mg/l dose of Zeatin.

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Introduction

Commonly known as medical mint or peppermint (*Mentha × piperita* L.), with 28 synonyms, is a perennial aromatic herbaceous plant species that is 30-90 cm tall, has erect and quadrangular stems, generally in shades of purple or purplish tones. Its leaves are light green, oval-shaped, oppositely arranged, short-stalked, and serrated edges, 4 to 5 cm in length, and its flowers are purple or reddish in color, usually surrounded by inconspicuous bracts in false racemes, the fruit of the plant consists of four ellipsoidal seeds (Singh et al., 2015). The species peppermint (M. × piperita) is native to Europe but is widely found in Eastern and Northern Europe, the United States, and Africa. However, it is cultivated worldwide (Singh et al., 2015).

M. × piperita is a species belonging to the Lamiaceae family, known as the mint family, and is a natural hybrid of *M. aquatica* and *M. spicata*. The Lamiaceae family comprises about 7,200 species and around 260 genera of trees and shrubs. The genus *Mentha* contains about 61 species and 13 natural hybrids (Benabdallah et al., 2018; Kumar, et al., 2011).

Mentha piperita encompasses a wide array of secondary metabolites across numerous groups. Among these, the principal ones include flavonoids (53%), phenolic acids (42%), different sub-groups such as lignans and stilbenes (2.5%), and terpenoids from monoterpenes (52%) and sesquiterpenoids (9%). Moreover, it is known that peppermint contains aldehydes (9%), aromatic hydrocarbons (9%), lactones (7%), and alcohol (6%) with menthone being the main component found in essential oils at a rate of (35–60%) (Mahendran and Rahman 2020). *Mentha piperita* is a widely used medicinal plant species proven by studies to exhibit antioxidant, antimicrobial, antiviral, anti-inflammatory, biopesticide, larvicidal, anticancer, radiation protective, genotoxicity, and anti-diabetic activities. It is used in folk medicine for fever, colds, mouth and throat inflammations, digestive system, anti-viral, anti-fungal treatment (Mahendran and Rahman 2020). The tea and essential oil derived from the leaves and flowers of medicinal mint are utilized in various fields.

Plant biostimulants are generally any substance (synthetic or natural) or microorganisms applied to plants in various forms and timings to increase nutrient content, improve abiotic stress tolerance, and/or enhance crop quality characteristics (Patrick 2015).

Zeatin belongs to a family of plant biostimulants called cytokinins and is known to play significant roles in plant growth and development. It aids in breaking apical dominance, supports the growth of leaves, contributes to the formation of chloroplasts, slows down the aging process, promotes seed germination, and helps regulate the cell cycle (Havlicek et al., 1997; Mok and Mok, 2001).

Kinetin, a member of the cytokinin biostimulant group, demonstrates effects such as delaying the aging process, promoting cell division, and thereby aiding in the plant's growth and development, regulating ethylene synthesis to slow down aging processes, and increasing chlorophyll synthesis (Toprak, 2019). Gibberellic acid (GA) is a phytobiostimulant produced by plants that plays crucial roles in various processes such as germination, water uptake, triggering of flowering, fruit development, shoot elongation, and metabolic functions. GA works in concert with other phytobiostimulants to regulate the development and growth of the plant (Zhu et al., 2019; Khan et al., 2020).

This study has been conducted to determine the effects of foliar applications of zeatin, kinetin, and gibberellic acid biostimulants at different doses on the growth and biochemical parameters of the medicinal mint plant during the seedling development phase.

Materials and Methods

Material

The study was conducted in the greenhouse within the Agricultural Sciences and Technologies Education Application and Research Center of Sakarya University of Applied Sciences, Faculty of Agriculture. The peppermint seedlings used in the research were procured from a commercial company.

Method

The trial was conducted with 3 replications according to the "Completely Randomized Experimental Design". In the study, peppermint seedlings were treated with zeatin (20, 40 mg/l), kinetin (50, 100 mg/l), and gibberellic acid (100, 200 mg/l) biostimulants, which are known to have effects on plant growth and development. A total of 21 pots with a capacity of 2 liters were used in the study. A homogeneous mixture prepared from finely sifted garden soil (3/4) and Klassman TS1 brand peat (1/4) was added to these pots. The ground where the pots were to be placed in the greenhouse for the study was leveled with a rake and then rolled, after which the pots were placed at a distance of 20 cm on top and 30 cm between rows.

After placing the pots in the greenhouse, five randomly selected pots were each watered with 500 ml of water. Plates were then placed under the bottoms of the pots to collect the water that drained through. After the drainage process was complete, an average of 215 ml of water accumulated in the plates from each pot. The water retention capacity of the pots was calculated by subtracting the drained water from the 500 ml of water added to each pot.their water-holding capacities were measured to be 285 ml. Subsequently, the seedlings were planted in the pots at an approximate depth of 3 cm on November 1, 2023. During planting, the lower two leaves of the plants were removed by hand if they touched the soil to prevent fungal disease. Throughout the experiment, each pot was watered with approximately 100 ml of water once a week.

Kinetin and gibberellic acid hormones were dissolved in 96% ethanol, while zeatin hormone was dissolved in NaOH and completed to 1 liter with distilled water. The prepared biostimulant solutions were filled into 1-liter spray bottles, wrapped in aluminum foil to protect them from light, and stored in the refrigerator. The first foliar applications of the biostimulants were made on 05.01.2024 (approximately 2 months after planting). The study being conducted during the winter months, growth and development were slower compared to the summer months, hence the delay in biostimulant application. Biostimulant applications were made by spraying approximately 10 ml onto the leaves of the seedlings in each pot. The biostimulant applications were carried out three times, at 4-day intervals. The trial was concluded on 19.01.2024, after measurements of plant heights were taken. The trial lasted for an average of 2.5 months. During the period from the setup to the conclusion of the experiment, it was determined that the average daytime temperature was 15°C, while the nighttime temperature was 4°C (Anonymous 2024). The roots of the plants were softened and separated with water from the soil. Then, the root lengths were measured and recorded with the help of a ruler. The fresh weights of the seedlings and roots were measured on a precision scale. The aerial parts of the seedlings and the roots were placed in drying paper and put in an oven at 35 °C for 108 hours to dry. Afterwards, the dry weights of the seedlings and roots were measured.

Total Phenolic Content Analysis

The total phenolic content was assessed using the Folin–Ciocalteu method according to Waterhouse (2002). Initially, 250 µL of Folin–Ciocalteu reagent and 50 µL of the extract solution were added to a tube, with the total volume adjusted to 3 mL using distilled water. Following a 5-minute incubation, 750 µL of 20% (w/v) Na2CO3 solution was added and mixed. The mixture was then left in the dark at room temperature for 90 minutes before measuring the absorbance at 765 nm with a UV-Vis spectrophotometer (Agilent Cary-60, Santa Clara, CA, USA). A gallic acid standard curve was generated by repeating the procedure with concentrations of 50, 100, 150, 200, and 300 µg/mL. The total phenolic content was expressed as gallic acid equivalents using the standard curve (mg GAE/100 g of dry weight thyme).

Determination of FRAP Reducing Capacity

Initially, 0.3 M sodium acetate buffer (pH 3.6), 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) solution, 20 mM $FeCl_3$, and 2 mM $FeSO_4$ solutions were prepared. The working solution was obtained by mixing the buffer solution, TPTZ, and $FeCl_3$ solutions in a 10:1:1 ratio. Absorbance measurements were taken at 593 nm using a 2 mM $FeSO_4$ solution to create the standard curve, followed by measuring the samples at a minimum of three different concentrations. The results were reported as mg extract/µmol Fe^{2+} equivalents (Sachett et al. 2021).

Determination of CUPRAC Reducing Capacity

This method was based on a partially modified version of a previously reported procedure. Plant extracts were taken in different concentrations (10, 20, 40 µg) into tubes. Then, 0.25 mL of $CuCl_2$ solution (0.01 M), 0.25 mL of ethanolic neocuproine solution, and 0.25 mL of CH_3COONH_4 buffer solution (1 M) were added. After incubating the mixtures in the dark for 30 minutes, absorbance values were measured at 450 nm against a blank (Ak and Gülçin 2008). The measurement results were evaluated by comparing them to trolox equivalents.

Statistical Analysis

Data analysis was conducted using COSTAT (version 6.03), and multiple comparisons were made using the Least Significant Difference (LSD) test at a 0.05 significance level.

Results and Discussion

Table 1 shows that the biostimulants applied during the seedling development stage of the peppermint plant have a statistically significant effect at the 0.05% level on all growth parameters. When examining Table 1, it can be seen that there are differences in growth parameters among the biostimulant applications. The highest seedling length was achieved with 17.70 cm from the gibberellic acid200 mg/l applications, the highest seedling fresh weight and root dry weight were achieved with 5.26 g and 1.34 g, respectively, from the zeatin40 applications, the highest root length was achieved with 33.25 cm from the kinetin50 applications, and the highest root fresh weight and seedling dry weight were achieved with 5.85 g and 0.94 g, respectively, from the kinetin100 applications, as shown in Table 1.

In bread wheat, foliar application of zeatin has been observed to shorten the maturation period, accelerate heading time, and increase main stem thickness. Additionally, it has been reported to increase spike length, spikelet number, grain weight per spike, and biomass yield, thereby enhancing agricultural productivity (Öztürk, 2023). It has been reported that the addition of zeatin to the medium in olive micropropagation increases callus formation compared to the control (Çiftçi, 2023). While zeatin40 application gave the best results in root dry weight compared to control and other applications, it gave higher results in seedling fresh weight compared to control and other applications. The increase in seedling fresh and root dry weights due to zeatin application from the leaf can be attributed to its promotion of cell division and growth, positive effects on the photosynthesis mechanism, and support for increased seedling and root development.

Studies conducted with different plants have reported that foliar applications of kinetin increase growth parameters (Tounekti et al., 2011; Tandel et al., 2018; Ghazy et al., 2023). Foliar applications of kinetin to *Ervatamia coronaria* plants have been reported to increase seedling fresh and dry weights, root fresh and dry weights, and root length compared to the control (Ashour et al., 2023). Kinetin biostimulant promotes the production of photosynthetic proteins by increasing chlorophyll content in plants, accelerates cell division, breaks apical dominance in plants, thereby increasing lateral branch and root formation, and consequently leads to an increase in both above-ground and below-ground biomass (Lazar et al., 2003; Bielach et al., 2017).

Foliar applications of GA3 have been reported to increase the height and quality of *Araucaria heterophylla* plants (Gul et al., 2006), increase seedling length in *Hibiscus sabdariffa* L. and wheat plants (Alharby et al. 2021), and enhance plant growth parameters when sprayed with 100 or 200 ppm GA3 in *Dahlia pinnata* plants (Yousef and Gomma, 2008), similar studies have been conducted by Santos et al. (1998) and Srivastava and Srivastava (2007). Application of gibberellic acid in thyme increases growth parameters, chlorophyll pigments, and volatile oil content (Dadkhah et al., 2016), and gibberellic acid applications have been reported to have positive physiological, morphological, and biochemical effects on plants (Taiz and Zeiger, 2010). The increase in growth parameters due to gibberellic acid applications in plants is reported to be associated with increased activity of enzymes such as carbonic anhydrase, nitrate reductase, and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) (Yuan and Xu, 2001; Afroz et al., 2005; Aftab et al., 2010). Additionally, GA3 is reported to stimulate cell growth and division, thereby supporting growth and development (Taiz and Zeiger, 2010). Previous study findings support our results.

It is seen in Table 2 that the applied biostimulants have a statistically significant effect on CUPRAC, FRAP and total phenolics at the 0.05% level. Table 2. The highest values for FRAP antioxidant activity and total phenolic compound parameters were obtained from Zeatin40 applications, with values of 1.72 mM/g AAE and 0.50 mg/g GAE, respectively (Table 2). The CUPRAC antioxidant activity value of 6.98 mM/g TE obtained from Kinetin50 applications stands out compared to other applications

Table 1. Effects of Some Synthetic Biostimulants on Growth Parameters of *Mentha piperita* Plants

Biostimulants	Seedling length (cm)	Seedling fresh weight (g)	Root length (cm)	Root fresh weight (g)	Seedling dry weight (g)	Root dry weight (g)
Control	15.73 ab	4.82 a	25.60 b	5.46 a	0.89 a	1.29 ab
Zeatin20	14.23 b	4.43 ab	30.50 ab	3.99 bc	0.67 bc	1.09 abc
Zeatin40	15.50 ab	5.23 a	31.50 ab	5.08 ab	0.81 ab	1.34 a
Kinetin50	14.17 b	4.55 ab	33.25 a	4.09 bc	0.65 bc	1.17 abc
Kinetin100	15.80 ab	3.46 b	29.00 ab	5.85 a	0.94 a	0.97 bc
Gibberellic acid100	14.30 b	4.65 a	30.25 ab	3.75 c	0.55 c	1.13 abc
Gibberellic acid200	17.70 a	3.94 ab	30.75 ab	5.16 ab	0.67 bc	0.93 c
LSD (0.05)	3.29	1.17	6.2	1.21	0.19	0.34
CV (%)	12.26	14.96	11.76	14.52	14.88	17.58

Table 2. Effects of Some Synthetic Biostimulants on Biochemical Parameters of *Mentha piperita*

Biostimulants	CUPRAC (mM/g TE)	FRAP (mM/g AAE)	Total Phenolics (mg/g GAE)
Control	5,10 bc	1,24 c	0,37 bc
Zeatin20	4,94 bc	1,32 bc	0,37 bc
Zeatin40	6,97 a	1,72 a	0,50 a
Kinetin50	6,98 a	1,63 a	0,48 ab
Kinetin100	6,36 ab	1,58 ab	0,46 ab
Gibberellic acid100	6,44 ab	1,55 ab	0,38 abc
Gibberellic acid200	3,66 c	1,20 c	0,28 c
LSD (0.05)	1,68	0,29	0,12
CV (%)	16,61	11,62	17,25

Table 3: Correlation Table of Some Synthetic Biostimulants on Investigated Parameters of *Mentha piperita*

	1	2	3	4	5	6	7	8	9
1	1								
2	-0.382	1							
3	-0.265	0.063	1						
4	0.718	-0.360	-0.533	1					
5	0.349	-0.197	-0.583	0.895**	1				
6	-0.391	0.920**	-0.125	-0.096	0.153	1			
7	-0.631	0.334	0.352	-0.186	0.067	0.488	1		
8	-0.472	0.241	0.526	-0.140	0.027	0.344	0.950**	1	
9	-0.492	0.271	0.330	0.047	0.325	0.492	0.923**	0.903**	1

* Correlation is significant at the 0.05% level; ** Correlation is significant at the 0.01% level; 1: Seedling Length, 2: Seedling Fresh Weight, 3: Root Length, 4: Root Fresh Weight, 5: Root Dry Weight, 6: Seedling Dry Weight, 7: CUPRAC, 8: FRAP, 9: Total Phenolic Content

Santos-Gomes et al. (2003) reported that doses of zeatin and kinetin hormones applied to medicinal sage increased both antioxidant activity and total phenolics compared to the control. Similarly, Ravanfar et al. (2020) reported that zeatin hormone applied to purple cabbage increased total phenolics and antioxidant activity compared to the control, while Yousaf et al. (2024) reported that zeatin hormone applied to corn plants increased antioxidant activity compared to the control. Günaydın et al. (2017) reported that kinetin hormone application in thyme plants increased total phenolic, flavonoid, and antioxidant parameters compared to the control, while Acidri et al. (2020) reported that kinetin hormone in coffee plants was effective in scavenging free radicals and increased both total phenolics and flavonoids as well as phenolic components compared to the control. Brenner and Schmulling (2012), Bhargava et al. (2013), Kocsy et al. (2013), and Reguera et al. (2013) stated that the effects of cytokinins, including zeatin and kinetin hormones, on plant development, especially on phenolic compounds and associated antioxidant defense systems, are dependent on the regulation of gene expression related to secondary metabolism, including flavonoid and phenylpropanoid biosynthesis, glutaredoxin, peroxidase, glutathione transferase, and antioxidant enzyme genes, under normal conditions or stress.

Table 3 shows a strong positive correlation at the 0.01% level between seedling fresh and dry weights, as well as between root fresh and dry weights. Additionally, root dry weight is positively correlated with CUPRAC and FRAP antioxidant activities and total phenolic content, and there is a positive correlation between FRAP activity and total phenolic content.

Conclusions

It has been observed that synthetic biostimulants applied foliarly have significant effects on plant growth, and that there are differences in hormones in terms of growth parameters. While gibberellic acid200 mg/l application stands out as the best application for seedling length, Zeatin40 application is prominent in increasing seedling fresh weight and root dry weight compared to the control. It has been observed that foliarly applied synthetic biostimulants have a significant positive effect on antioxidant activities, total phenolic content, and total carotenoid content. Particularly, while kinetin50 applications show high CUPRAC antioxidant activity, zeatin40 applications have been the most effective biostimulant in terms of FRAP antioxidant activity and total phenolic content. These findings indicate that hormone applications may contribute to increasing plant health and antioxidant capacity, enhance growth efficiency through synthetic biostimulant use, and be beneficial in optimizing growth parameters. However, in order to make sustainable recommendations for the hormones applied, it is recommended to perform residue analyses, dıfferent doses and field trials.

Declarations

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