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Investigating the Effect of Plant Regulators along with Potassium Nitrate on Increasing the Germination Capacity of *Malva sylvestris* Seeds

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A B S T R A C T

To investigate the effects of plant regulators along with potassium nitrate on the germination characteristics of Mallow (*Malva sylvestris*) plant, an experiment was conducted using a factorial management based on a completely randomized design with three replications in the physiology laboratories of the Khorasan Razavi Agricultural and Natural Resources Research and Education Center in 2024. The first factor consisted of priming treatments at four levels [GA_3 (Gibberellic Acid), BA (Benzyladenine), NAA (Naphthalene Acetic Acid), and PN (Potassium Nitrate)], the second factor involved different levels of the first factor at seven concentrations (0, 100, 200, 300, 400, 500, and 1000 ppm) and the third factor included two light conditions (light and dark). The results indicated that the highest germination percentage (GP) was obtained from 500 mg l^{-1} of GA3, followed by PN at 200 mg l^{-1}, which recorded a GP of 28.7%. The highest germination rate (GR) was obtained from the application of GA3, while the lowest rate for this trait was observed with PN. In both dark and light conditions, the lowest GR was observed due to the effect of BA, while its maximum was recorded from PN under dark conditions. The highest seedling length was observed with GA3 under the dark conditions. The highest rootlet length was obtained with the PN treatment (25.9 mm) under the dark conditions. The highest SVLI value (10.15) was recorded with the application of 200 mg.l^{-1} of PN, representing 26% increase compared to the control within the same treatment. All concentrations of BA had the lowest attributed traits in the germination. Overall, the results showed that the use of PN and GA3 are suitable options for enhancing the germination capacity of *M. sylvestris* plant seeds.

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Introduction

One of the main features that determines both the economy and ecology of plant growth is success in seed germination and the establishment of normal seedlings (Nonogaki et al., 2010). In the plant life cycle, germination is considered the most critical phase. This phase begins with the uptake of water and leads to the protrusion of the radicle and seed development. Germination is a complex process that can be divided into three phases: (I) imbibition, (II) respiration and (III) cell division (Bhatla and Lal, 2023). In phase I, the seed rapidly absorbs water, which leads to swelling of the seed and the initiation of metabolic activities, activating the essential biochemical reactions for germination. The phenomena of hydrolysis, biosynthesis of molecules, and mobilization of stored reserves such as starch, proteins, and lipids occur in seeds. Following these events, the necessary energy and buildings for seed growth and development are provided (Ali and Elozeiri, 2017). In phase III, rootlets appear as a result of cell division and elongation, indicating the final stage of germination (Nautiyal et al., 2023). Seeds can be classified into two categories: orthodox seeds, which survive drying and/or freezing during off-site storage. Their ability to withstand desiccation and storage varies, and some of them are more sensitive than others. So some seeds are considered moderate in storage, while others are quite orthodox (Walters, 2015). In the second category are recalcitrant seeds, which do not survive drying and freezing during in situ preservation. Generally, these seeds cannot withstand the effects of drying or temperatures below 10°C; therefore, they cannot be stored for long periods like orthodox seeds because they may lose their viability (Barbedo, 2018). The seeds of most species of medicinal plants exhibit dormancy to adapt ecologically to environmental conditions and belong to the orthodox category. There are three levels of dormancy: population level, single-plant level, and finally, individual progeny seed level (Penfield, 2017). Thus, it is necessary to diagnose the mechanisms that affect breaking dormancy and induce suitable conditions for germination. Several factors such as light, phytohormones, chemical substances, boiling water, and mechanical or chemical scarification, influence seed germination, leading seed to transition from a dormant state to active growth. Some seeds can germinate only when they receive an adequate amount of light, while others need shade or darkness. Light signals are detected by photoreceptors (phytochromes, phototropins) in seeds, causing changes in the expression of light-regulated genes involved in hormonal biosynthesis, enzyme activation, and seed reserve mobilization (Lau and Deng, 2010). Consequently, these receptors undergo a conformational change, triggering a series of biochemical events that activate the genes involved in germination. Nitrogen is an essential macronutrient for plants because it is a key structural component of macromolecules. The primary nitrogen source for plants is nitrate, which serves as a signaling molecule to promote seed dormancy release and stimulate seed germination in many plant species (Alboresi et al., 2005). Founding researchers demonstrated that applying exogenous nitrate to the germination medium stimulates dormancy release and promotes germination in arabidopsis (Yan and Chen, 2020). The study by Ghasemi Pirbaluti et al. (2008) showed that potassium nitrate and gibberellic acid (GA3) treatments had the most positive effects on breaking dormancy and promoting seed germination in thyme, hyssop, and anise seeds. In another study, Yazdanipour et al (2017) reported that applying potassium nitrate (0.1 mol 68%) and GA3 (100 ppm) significantly improved seed germination and effectively broke the dormancy of knapweed (*Centaurea balsamita* Lam.). Following the activation of signal interplay among endogenous phytohormones by environmental factors such as light, temperature, and nitrate, the physiological activities within seeds are regulated (Seo et al., 2009). GA3, as a phytohormone, plays the most crucial role in mediating the light and temperature-induced transition from seed dormancy establishment to seed germination. During the seed imbibition phase, the expression of biosynthetic GA3 genes is strongly induced in the cortex and endodermis of the embryonic axis of germinating seeds, resulting in the de novo synthesis of GA3 in the embryo (Holdsworth et al., 2008). The synthesis of hydrolytic enzymes induced by GA3 in the seed facilitates the breakdown of the endosperm and seed coat, thereby mobilizing seed storage reserves in the endosperm and accelerating embryo growth and radicle protrusion (Finkelstein et al., 2008).

Mallow *(Malva sylvestris* L.) is a perennial plant with a height of 100 to120 centimeters. This plant is native to Europe, North Africa, and Southwest Asia. *M. sylvestris* is an important medicinal plant both in Iran and globally. It contains a variety of chemical compounds, including mucilage, tannins, leuco-anthocyanins, anthocyanins, linoleic acid, palmitic acid, linolenic acid, oleic acid, phenols, and terpenoids. It possesses several beneficial properties, including high antioxidant capacity, pain relief, anti-inflammatory, and antiseptic (Tabaraki et al., 2011). The seeds of this plant have physical dormancy due to their hard seed coat, which is usually broken by scratching or other natural factors. Therefore, the seeds can remain dormant in the soil for extended periods and may germinate sporadically in different seasons once the seed coat becomes permeable through an unknown process. However, this type of dormancy results in either the impermeability of the seed coat to water and/or gases, the mechanically prevention of radicle extension, or the seed coat preventing the release of inhibitory substances from the embryo or supplying inhibitory compounds to the embryo (Baskin and Baskin, 2004). Veiga-Barbosa et al. (2016) found that among the various treatments for breaking seed dormancy of *Malvella sheradiana* L. (including mechanical scarification, cold stratification, liquid nitrogen, freezing, hot water, dry heat, gibberellic acid, and sulfuric acid), gibberellic acid (1000 $mg.l^{-1}$) increased germination percentages, but sulfuric acid was the most effective treatment. As previously mentioned, although the seed dormancy of the Malva plant is physical, priming the seeds with chemical compounds can prolong the first stage of water absorption (the imbibition phase), leading to increases in the permeability of the seed coat (Ruttanaruangboworn et al., 2017). During this stage, the activities of enzymes involved in starch hydrolysis and the strength of the roots and stems increase, ultimately leading to germination and the development of strong seedlings (Abnavi and Ghobadi, 2012).

Understanding the ecology of plant germination and sprouting plays a significant role for managing the production of seed-propagated crops. Therefore, this study aimed to evaluate the effects of various chemical compounds, including plant growth regulators (gibberellic acid, naphthalene acetic acid, and benzyladenine) and potassium nitrate on seed germination under both light and dark conditions.

Materials and Methods

To investigate the effects of plant regulator substances and potassium nitrate on the germination characteristics of *M. sylvestris* plant, an experiment was conducted as a factorial based on completely randomized design with three replications in the physiology laboratory of the Khorasan Razavi Agricultural and Natural Resources Research and Education Center in 2024. The first factor was priming treatment with four levels [GA3, BA (Benzyladenine), NAA (Naphthalene acetic acid), PN (Potassium Nitrate)], the second factor consisted of seven levels of the first factor (0, 100, 200, 300, 400, 500, and 1000 ppm), and the third factor included two germination conditions (light and dark). To prepare the hormones treatment, GA3, NAA, and BA hormones were first weighed (0.1, 0.2, 0.3, 0.4, 0.5, and 1 g). Each concentration was then poured into a separate test balloon, and the solvent (70% ethanol) was added drop by drop until the hormone was completely dissolved. Then, distilled water was added to the test balloon until it reached a volume of 1000 ml. The balloon was then shaken well, and six concentrated stock solutions were prepared for three hormones (GA3, NAA, and BA). To prepare different concentrations of potassium nitrate, it was done according to the standard instructions. The required hormones and PN concentrations were poured into the Petri dishes containing 25 seeds arranged in a 5*5 pattern. The seeds were obtained from the Forest and Pasture Department of the Research Center. First, the seeds were purified and disinfected in a 1% sodium hypochlorite solution for 5 minutes, followed by washing with distilled water. The 25 seeds were placed on a layer of Whatman filter paper number 1 inside each glass Petri dish with a diameter of 9 cm, and the filter papers were then moistened with seven ml of the treatment solutions**.** After planting the seeds in the

Petri dishes and applying the treatments, the lids of the Petri dishes were completely sealed with paraffin glue and placed in the germinator at room temperature. The temperature of germinator chamber was adjusted to 25°C±2, and the relative humidity was maintained at 50-60%. The lighting conditions consisted of 12 hours of daylight and 12 hours of darkness. During the 10-day experiment, the number of germinated seeds was recorded daily, and at the end of the experiment, the lengths of the roots and seedlings were measured. The percentage of germination and the germination rate (GR) were calculated using equation 1 (Fang et al., 2006) and equation 2 (Verma et al., 2005), respectively, while the seedling vigor length index (SVLI) was obtained from equation 3 (Savaedi et al., 2024).

$$GP\% = (\Sigma n/N) \times 100 \quad (1)$$

Where GP: germination percentage, n: number of germinated seeds per day, N: total seeds planted in each replicate.

$$GR = \Sigma (Ni/T_i) \quad (2)$$

Where Ni: number of germinated seeds per count, Ti: the number of days is counted from the beginning

$$SVLI= ((GP \times SL)/100) \quad (3)$$

Where GP: germination percentage, SL: seedling length.

The analysis of variance (ANOVA) was performed using MSTAT-C and Minitab Ver. 16 software, and means were compared with Duncan's multiple range test (DMRT) at a 5% probability level.

Results and Discussion

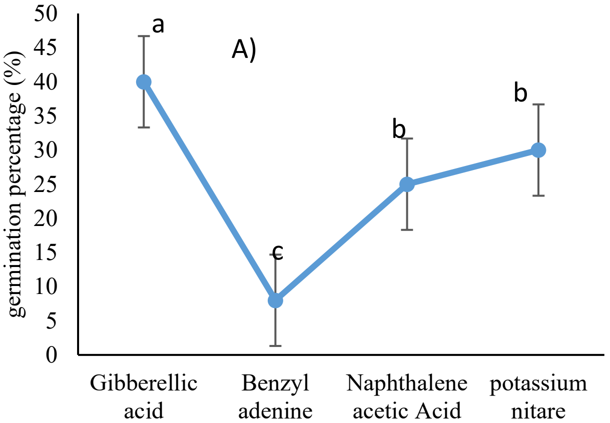
Effect of Hormonal and Potassium Nitrate Treatments

Different experimental treatments had a significant effect on the germination percentage (GP) (Table 1). The highest germination percentage was obtained from the GA3 treatment, while the lowest was observed with the benzyladenine (BA) treatment. The GA3 treatment resulted in a GP of 40%, which was 15% higher than that of the other treatments (Figure 1a). The results of Veiga-Barbosa et al. (2016) indicated that applying a GA3 solution (1000 $mg.l^{-1}$) increased the germination percentages of *Malvaella sheradina* plants across all tested soaking times in sulfuric acid. The highest germination rate (GR) was obtained from the application of GA3, while the lowest GR was observed with PN (Figure 1b). Tombegavani et al. (2020) reported that the highest GR was recorded from the application of 500 ppm GA3, which also produced the highest GP among all hormonal treatments. Seed germination requires favorable environmental conditions, including water, oxygen, temperature, and exogenous matter, along with a combination of multiple cellular processes such as synthesis, transport, signaling, sensing, and hydrolysis (Miransari and Smith, 2014). It appears that the exogenous application of the GA3 hormone effectively stimulates seed-hydrolyzing enzymes, particularly alpha-amylase. This enzyme catalyzes the hydrolysis of internal α-1, 4-glycosidic linkages in starch into low molecular weight products, such as glucose, maltose, and maltotriose units, which are used as an energy source for primary root growth.

Table 1. The source of variation, degree of free and mean of squares of germination rate and percentage, length of rootlet and seedling.

SOV	df	GP	GR	LRL	LS	SVLI
Hormonal and Potassium nitrate Treatments (HPT)	3	118**	0.011**	148.2**	34.1**	72.1**
Concentration HPT (Con_HPT)	6	482.3*	0.019*	211**	108.1**	98.7**
Experimental of Condition (EC)	2	845**	0.00011ns	345**	261**	18ns
HPT× Con_HPT	18	58.3**	0.0014**	66.9**	48.2**	117**
HPT × EC	6	14.5ns	0.00018ns	44.3ns	22.1ns	24.6ns
Con_HPT × EC	12	412**	0.010**	205**	157**	112**
HPT × Con_HPT × EC	36	11.2ns	0.00005ns	17.1ns	19.5ns	21.5ns
Error	72	6.05	0.0001	11.2	15.4	13.1
CV (%)		8.55	11.5	14.3	13.1	8.14

GP: Germination percentage; GR: Germination rate; LRL: Length of root lets; LS: Length of seedling; SVLI: Seedling Vigor Length Index; ^{Ns}, *and**are non-significant and significant at the 5 and 1% probability levels, respectively



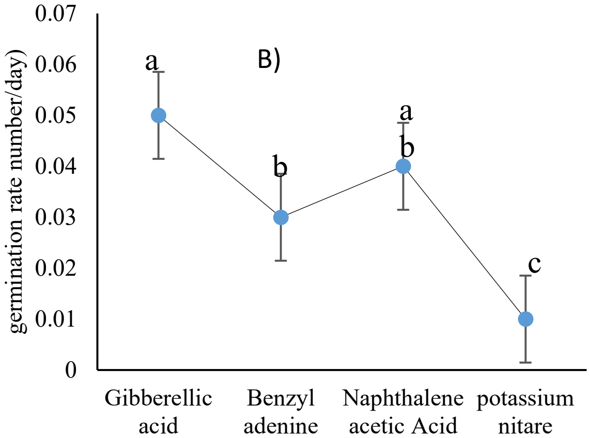


Figure 1. The effect of hormonal treatment on germination percentage (A) and germination rate (B)

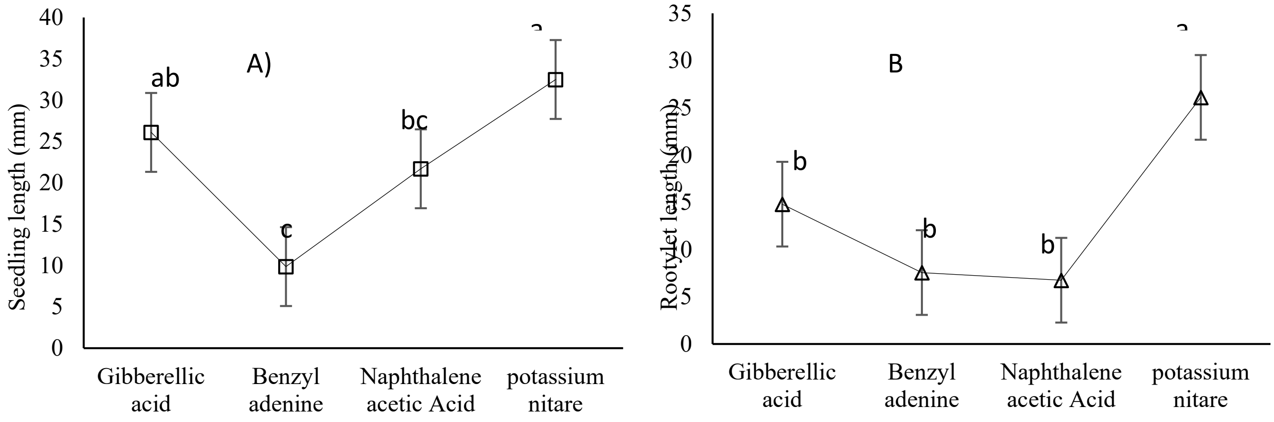


Figure 2. The effect of hormonal treatment on seedling (A) and rootlet (B) length.

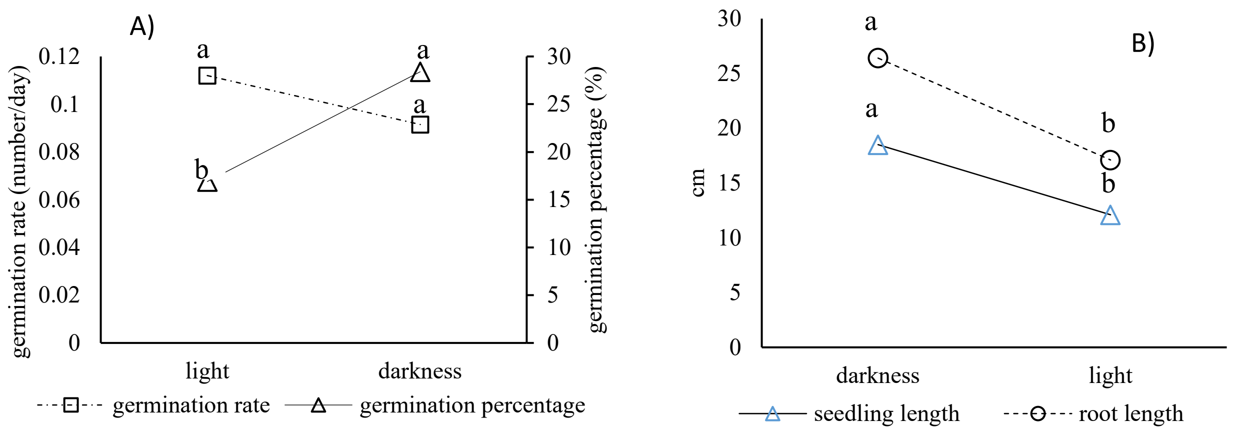


Figure 3. The effect of light and dark treatments on (A) germination percentage and rate (B) length of seedling and rootlets.

The results of the analysis of variance revealed that the type of hormonal and PN substances used for seed priming significantly affected both the length of the seedling and the rootlets (Table 1). The lengths of the seedlings were 26.1, 9.87, 21.7 and 32.5 mm for the application of GA3, BA, NAA and PN, respectively (Figure 2a). The effect of potassium nitrate on rootlet length was very impressive compared to the other treatments. The effect of seed priming compounds on rootlet length was similar to that on seedling length, as rootlet length increased due to the applied potassium nitrate treatment by about 43%, 71%, and 74% in comparison to that of GA3, BA, and NAA, respectively (Figure 2b). The highest seedling vigor length index (SVLI) of 10.7 was obtained from GA3, whereas the lowest value for this trait (0.696) was recorded with BA. The results of Kabilan et al. (2022) showed that the prime seed treatment with potassium nitrate, gibberellic acid, naphthalene acetic acid, and control in the plant caused an increase in the lengths of the seedling and rootlet of the mundu chilli (*Capsicum annuum* L.), so that the lengths of the seedlings were 15.6, 16.8, 15.2 and 14.2 cm, respectively, and the rootlets lengths were 6.30, 6.80, 5.60 and 4.50 cm, respectively. The researchers reported that potassium nitrate through (a) reducing the endogenous levels of abscisic acid in embryos (Bhatt et al., 2019), (b) causing vacuolation and weakening of the cell wall, which can be effective in water absorption and elongation (Bethke et al., 2007), and finally (c) increasing the acidification of NADPH and ATP compounds stimulates the pentose phosphate pathway in the cell. During this process, certain fatty acids are produced and ribose is synthesized for the formation of nucleotides and nucleic acids (Finkelstein et al. 2008), which ultimately leads to cell proliferation and increases its length, enhancing both the seedling and the rootlet.

Experimental Conditions (Light and Darkness)

The means comparison indicated that the percentage of germination increased under dark conditions, showing a significant difference compared to light conditions (Table 1 and Figure 3a). There was no significant difference in germination rate (GR) between dark and light conditions; however the germination rate was higher in light than in darkness (Figure 3b). The results of the analysis of variance showed that the environmental conditions significantly affected both rootlet and seedling length (Table 1). The length of both rootlet and seedling were greater in the dark than in the light conditions, with rootlet and seedling lengths measuring 26.6 and 18.5 mm, respectively, in the dark conditions (Figure 3b). The value of SVLI was not affected by germination conditions; however, the value of this index under dark conditions was approximately 0.5 units higher than that under light conditions, with the value of the index in light conditions being 3.01. Light signals are detected by photoreceptors in seeds, including phytochromes, cryptochromes, and phototropins. These photoreceptors play an important role in integrating light information and controlling germination responses (Voitsekhovskaja, 2019). Most plant seeds are not sensitive to light; however, seeds found in forest areas will not germinate until sufficient light is available for seedling growth (Gallagher, 2013). Light spectrums have different effects on seed growth and development. For example, blue light and UV light can influence seed germination; however, their effects are more complex and vary depending on the plant species and environmental factors (Fantini and Facella, 2020). Phytochrome is synthesized when a seed is exposed to red light (600-760 nm). This photoreceptor converts to Pfr, which triggers a series of biochemical events that activate genes involved in germination (Higuchi and Hisamatsu, 2016). When seeds are exposed to far-red light, phytochromes undergo a distinct conformational change that may counteract the effects of red light and inhibit germination. This phenomenon is known as phytochrome-mediated inhibition of seed germination (Smith, 2000). The results of Chandra et al. (2024) comparing different lights conditions on the germination of *Nepenthes mirabilis* and *Cyrtopodium glutiniferum* showed that the highest GP was observed under yellow light, followed by red light, while dark conditions had no effect on GP. Aref (2002) studied the effect of light intensity at levels of 100%, 50%, and 25% on seed germination. The results of the study indicated GP of 67%, 45%, and 33% for *Delonix regia*, *Cassia fistula* and *Enterolobium saman*, respectively. In another study, Zucareli et al. (2009) demonstrated that light/dark conditions had no significant effect on the germination of *Passiflora nitida* plant. Additionally, some types of seeds can germinate only when they receive an adequate amount of light. Meanwhile, other plants have mechanisms to determine whether they are in the shade of neighboring vegetation or direct light source; this process is initiated by photoreceptors in the seeds.

Interaction between Treatments

Interaction between experimental conditions and hormonal and potassium nitrate treatments

The germination percentage (GP) and germination rate (GR) were not affected by the interaction of chemical substances under the experimental conditions (Table 1). The lowest GP in dark conditions was related to BA hormone treatment, while the highest was associated with GA3 hormone. The reaction process of the GP under light conditions was similar to that in dark conditions. Under light conditions, the BA hormone exhibited the lowest GP at approximately 5%, while the highest GP was observed with GA3 with a value of 19% (Figure 4 a). The results of the analysis of variance indicated that the lengths of the seedlings and rootlets were not affected by the interaction between the experimental conditions and the chemical substances (Table 1). Regarding rootlet length, the maximum measurement was recorded from potassium nitrate treatment (35.9 cm) under dark conditions, whereas the minimum was observed with BA hormone (5.32 cm). The results showed that under dark conditions, the seedling length was approximately 32.2 cm with the GA3 hormone, representing an increase of approximately 34% compared to light conditions. This trend was also observed in the other two hormones under light conditions; however in the potassium nitrate treatment under light conditions, the seedling length was approximately 28% greater than that observed under dark conditions (Figure 4b). The results indicated that the interaction between experimental conditions and hormonal and potassium nitrate treatments had no significant effect on the SVLI index (Table 1). However, the highest SVLI value was obtained from the effect of the GA3 hormone in dark conditions with a value of 6.46 units, while the lowest value of this index (0.728) was obtained from the effect of the BA hormone in both light and dark conditions (Figure 4c).

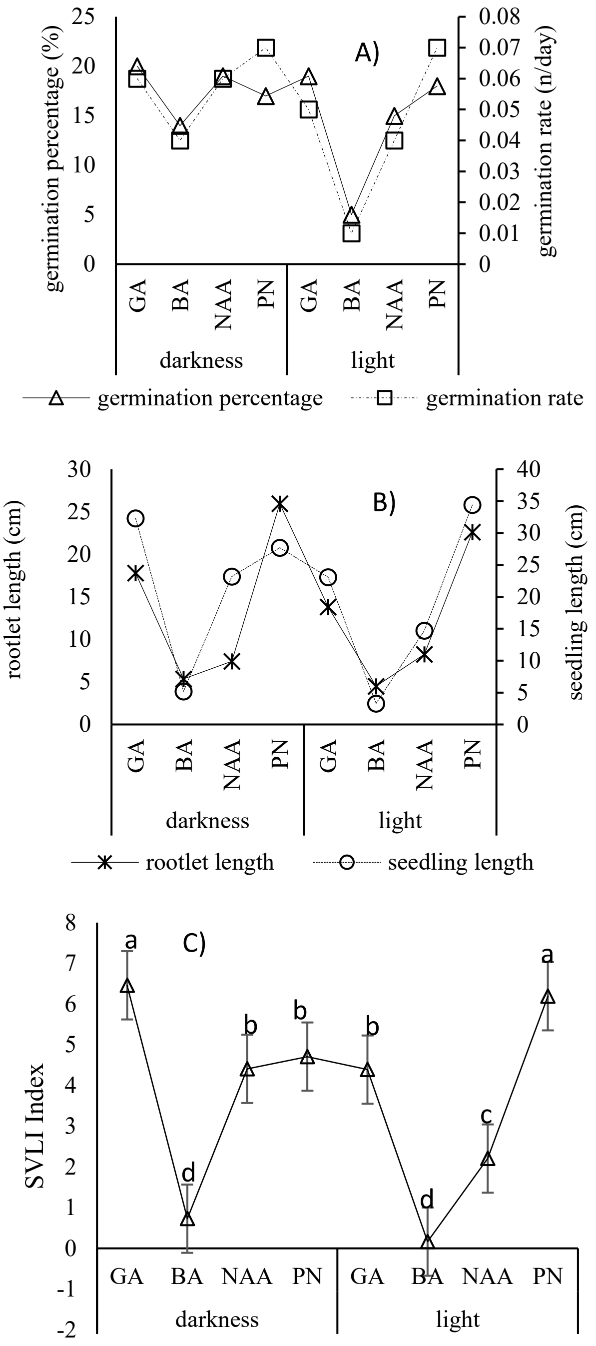


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Figure 4. The interaction effect between treatments and light on (a) germination percentage and rate (b) length of seedling and rootlets and c) seedling vigor length index (SVLI). GA: gibberellic acids, BA: benzyladenine, NAA: naphthalene acetic acid, PN: potassium nitrate.

Interaction between concentration and type of hormonal and potassium nitrate treatments

The results showed that the lowest percentage of germination was recorded with the application of BA hormone concentrations of 200, 300, 400 and 1000 mg.l^{-1} with values of 2.51, 2.65, 3.64, and 2.65%, respectively, and the highest amount of GP was obtained from of the level of 500 mg.l^{-1} of GA3. It is worth mentioning that the trend of the effect of different concentrations of the experimental treatments on the germination rate (GR) also followed the trend of the GP (Figure 5a).

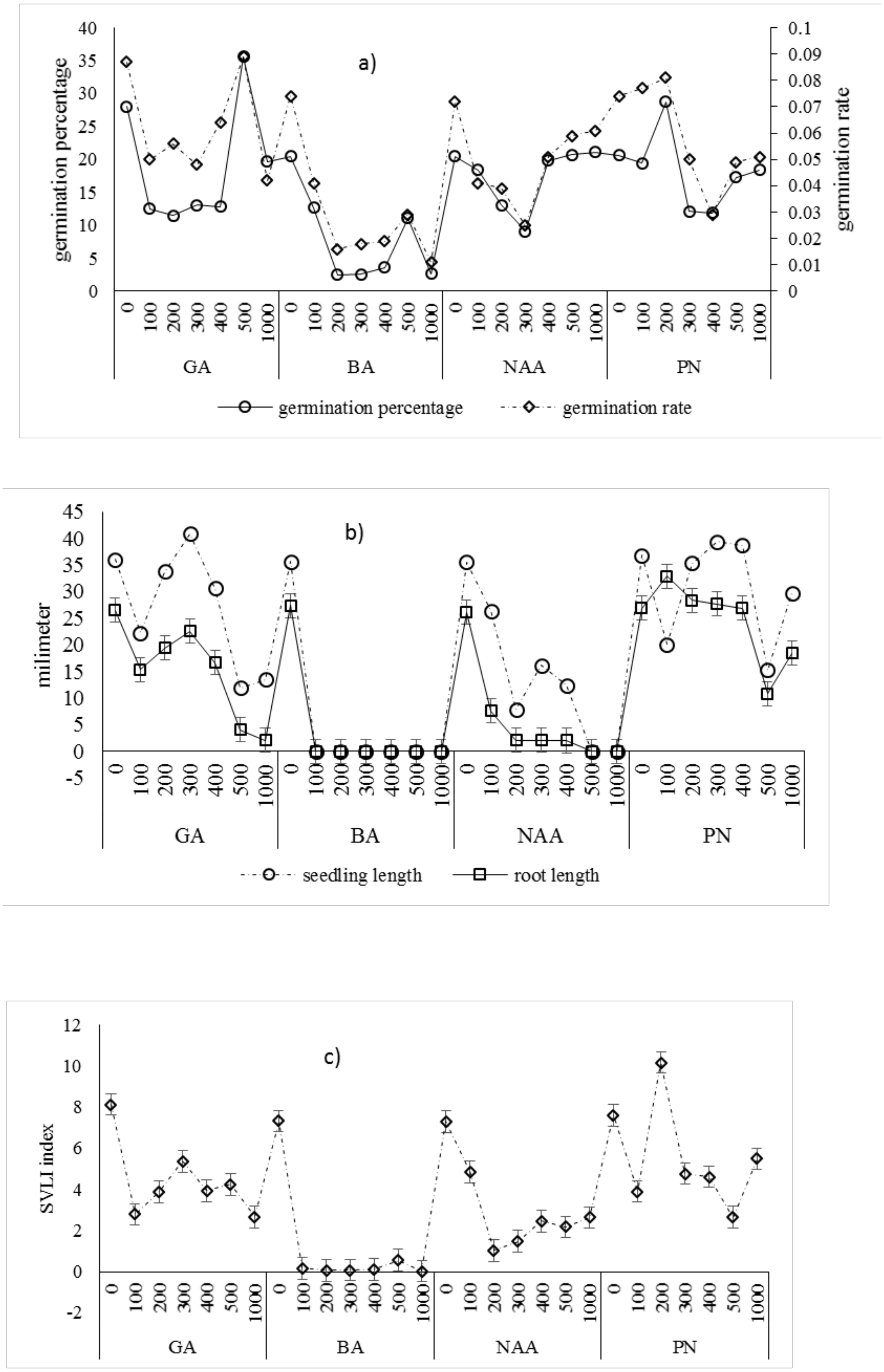

Figure 5. The effect of experimental treatments on a): germination percentage and rate, b) seedling and rootlet length, c) seedling vigor length index (SVLI).

The comparison of means revealed that the maximum seedling length was obtained from the application of 300 mg.l^{-1} GA3, and the highest rootlet length was recorded from the application of potassium nitrate at concentrations of 100 and 200 mg.l^{-1} with values of 32 and 28 mm, respectively. In the traits of rootlet and seedling length, the BA hormone treatment showed the lowest values (Figure 5b). The seedling vigor length index (SVLI) was affected by the interaction between concentration and type of experimental treatments. The highest SVLI value (10.15) was recorded with a concentration of 200 mg per liter of PN, representing a 26% increase over the control in the same treatment. The lowest SVLI value was recorded from the application of 500 mg.l^{-1} of BA hormone; however, all concentrations of BA hormone had the least SVLI (less than 1 unit) (Figure 5c). The results of the study by Savaedi et al. (2024) showed that varying concentrations of the cytokinin hormone significantly affected the SVLI of black seed plant, with the lowest (0.62) and highest (5.14) values of this index obtained from the concentrations of 80 and 10 mg.l^{-1} of cytokinin, respectively. The trend of the effect of the type and concentration of hormone in our research is consistent with the aforementioned studies, but it seems that the increase in the BA concentration in the current experiment has led to a sharp decrease in SVLI. Numerous physiological and biochemical processes in plants are regulated by plant hormones including abscisic acid (ABA), ethylene, gibberellins, auxin (Indole-3-Acetic Acid; IAA), cytokinins, and brassinosteroids.

Food reserves including proteins, lipids, starchs, and nutrients must be available through specific pathways and enzymatic activities for the seed to germinate (Miransari and Smith, 2009). Proteins are also stored in the root and seedling radicle (Tiedemann et al., 2000). Storage proteins do not move simultaneously within various parts of the seed. Carboxypeptidase and aminopeptidase are enzymes that are activated during protein transport. Cystatins, also known as phytocystatins, are a group of proteins that inhibit the activity of cysteine proteinases. They serve as inhibitors of protein degradation and play a regulatory role during seed germination in plants. Molecular changes, including alternations in proteins and hormones, as well as the balance between abscisic acid and gibberellins, are the most important parameters controlling the seed germination process. Consequently, it seems that the production and activity of plant hormones are regulated by the expression levels of corresponding genes. Accordingly, variations in the germination of different seed cultivars related to their gene complement (Miransari, 2012). The external application of plant hormones and chemical compounds to enhance the germination capacity of seeds induces changes in the hormonal balance within the seeds, which subsequently leads to alterations in gene expression levels. As a result, this can affect the activation or deactivation of enzymes that are effective in germination. Furthermore, the decrease in germination indicators caused by specific hormone concentration levels can be attributed to this factor.

Conclusion

It is crucial to identify the mechanisms that influence the breaking of dormancy and induce conditions suitable for germination. Several factors e.g. light, phytohormones, chemicals, boiling water, and mechanical or chemical abrasion, can faciliate the transition of seeds from dormancy to active growth. The findings of our study indicate that the application of various hormonal and potassium nitrate treatments significantly affects germination percentage and rate, as well as rootlet and seedling length, and seedling vigor length index. Therefore, the application of potassium nitrate at a concentration of 200 mg.l^{-1} or GA3 hormone at concentrations ranging from 200 to 300 mg.l^{-1} causes an increase in the germination indices of the *M. sylvestris* plant.

Declarations

Ethical Approval Certificate

This research does not involve any data or materials related to humans, animals, or other living organisms. Consequently, ethical committee approval and informed consent is not required for this study.

Author Contribution Statement

S.F.F.K.: Data analysis, formal analysis, and writing the original draft

N.B.: Data collection, conceptualization, methodology, and review

Fund Statement

Not applicable

Conflict of Interest

The authors declare no conflict of interest.

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