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Increasing the resistance of the hollyhock plant to cold stress: silicon or selenium

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ABSTRACT

It is well-known that freezing affects crop growth worldwide and significantly reduces crop production in the affected regions. The study objective was to investigate the effect of sodium silicate and sodium selenite individually or in combination on *Alcea rosea* plants grown under cold temperature stress. In this work, hollyhock plants were grown with sodium selenite and sodium silicate to 1/2 Hoagland solution, including 1) control, 1 mM sodium sulfate, 2) 1 mM sodium silicate 3), 0.03 mM sodium selenite, 4) 1 mM sodium silicate + 0.03 mM sodium selenite and exposed to non-freezing (20 °C) and freezing temperature (−4 °C). Nutritional elements, photosynthesis parameters, electrolyte leakage, malondialdehyde, water content, osmolytes, and antioxidant enzymes were determined. The application of Si + Se effectively enhanced the Si and Se content of leaves (54% and 164%) compared to control plants. The combined Si and Se addition increased phenols, water-soluble sugars, proline, chlorophyll, net photosynthesis (*Pn*), and stomatal size under cold stress. Moreover, Si and Se in combination in stressed plants inhibited electrolyte leakage and malondialdehyde (MDA) by increasing the activities of enzymes such as superoxidase dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT). The maximal to minimal values of essential oil content, relative water content (RWC), and stomatal density depended on the treatment: Si + Se > Si = Se. By increasing K content, phenol, proline, and water-soluble sugars, Si + Se > Si > Se application decreased the adverse effects of cold stress on the dry weight of the hollyhock.

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Essential oil; freezing stress; medicinal plant; sodium selenate and silicate

Introduction

The Alcea genus is a group of medicinal plants with ornamental potential. It is a part of the Malvaceae family and consists of around 60 species, mainly found in the East Mediterranean region. Hollyhock, scientifically known as *Alcea rosea* L., is a popular plant commonly found in gardens and parks, playing a significant role in urban areas. Several pharmacological studies have highlighted the anti-inflammatory, antibacterial, and analgesic properties of this plant. In traditional medicine, the roots of *Alcea rosea* are utilized to treat various ailments such as bronchitis, diarrhea, constipation, severe cough, and angina (Khosravan et al. 2017; Zhang et al. 2015). However, its most notable effect is its diuretic action, making it a remedy for dysuria, retching, and kidney stones (Ahmadi et al. 2012).

Temperature is an important abiotic factor that determines plant performance and geographic distribution. Cold stress affects the metabolism and distribution of tropical and subtropical plants.

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Freezing temperatures can have detrimental effects on plants, particularly those that are not adapted to cold climates. When temperatures drop below freezing, ice crystals can form within plant cells, causing physical damage and cell death. This can result in visible symptoms such as wilting, browning, and necrosis in sensitive tissues like leaves, flowers, and young shoots (Adhikari et al. 2022). Additionally, freezing temperatures can inhibit photosynthesis and reduce the plant's ability to produce energy and synthesize carbohydrates. Stomatal closure, a protective mechanism against freezing, can limit gas exchange and carbon dioxide uptake, further impacting the plant's growth and development (Kazan and Lyons 2016).

Recently, there has been an adoption of diverse chemical, physical, and biological techniques to achieve stable crop productivity in cold conditions. Among these approaches, the external application of nutrients has emerged as a highly effective method to mitigate the negative impacts of cold stress. While silicon (Si) is not considered an essential element for higher plants, it is widely recognized for its beneficial effects on promoting healthy growth and development, especially when plants face various biotic and abiotic stresses. Si plays a role in enhancing plant tolerance to stresses such as cold stress, fungal diseases, salt stress, and drought stress, partly due to its ability to enhance antioxidant defense capacity (Zargar et al. 2019). Previous studies investigating Si uptake and transport in wheat have shown that leaves of Si-treated plants grown hydroponically at low temperatures (0 °C–4 °C) exhibit increased tolerance to cold-induced wilting, and the roots demonstrate improved nutrient absorption, indicating Si's ability to confer resistance and/or tolerance to chilling or freezing stress (Liang et al. 2008). Recent research has reported that freezing stress significantly inhibits wheat plant photosynthesis and water use efficiency, but the addition of Si has been shown to significantly enhance these processes (Joudmand and Hajiboland 2019). Interestingly, in Si-amended freezing treatments, the content of soluble sugar and proline in seashore paspalum (*Paspalum vaginatum* Swartz) increased significantly compared to non-Si-amended freezing treatments (He et al. 2010).

Selenium (Se) is a vital trace element that has been acknowledged to have numerous beneficial effects on plants, even at lower concentrations. It acts as a strong phytoprotectant, safeguarding plants from various environmental challenges (Feng, Wei, and Tu 2013). When applied, Se can enhance tolerance to abiotic stresses either alone or in conjunction with other plant nutrients. It accomplishes this by regulating water usage, photosynthesis, and sodium (K^+) balance, thereby promoting overall growth. It activates antioxidant defense mechanisms, including GPX, APX, DHAR, MDHAR, CAT, POD, and SOD activities, resulting in reduced levels of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) during cold stress (Chongping, Wenjie, and Junlin 2022). These antioxidants work together to decrease lipid peroxidation, maintain cell membrane integrity, and preserve concentrations of photosynthetic pigments (Cao et al. 2022). Interestingly, Se has been shown to enhance growth attributes, photosynthetic ability, and antioxidant defense in crops, particularly in wheat, when faced with chilling stress (Chu, Yao, and Zhang 2010). The positive effects of Se on plant growth are primarily attributed to its antioxidative properties, leading to increased accumulation of soluble sugars and proline in shoots, higher leaf relative water content, biosynthesis of photosynthetic pigments, and enzymatic upregulations that help maintain metabolic balance in plants experiencing cold stress (Liu et al. 2021). Currently, there is a lack of further information in the literature regarding the interactive effects of Si and Se on freezing stress in higher plants and the underlying mechanisms involved.

However, information on the separation or interaction of Si and Se to increase cold tolerance in ornamental plants has been limited. We hypothesize that the alleviation of freezing injury by silicon (Si) and selenium (Se) may be due to their ability to enhance antioxidant defense activities. This, in turn, leads to a reduction in oxidative damage to cell membranes by improving water content in leaf tissues. This mechanism has been previously proposed to explain how Si and Se alleviate cold stress in plants. The specific objectives of the study were to (1) investigate the effects of cold stress on hollyhock biomass, (2) examine how Si and Se affect the physiological

response of plants to cold stress, and (3) provide scientific knowledge on the abilities of hollyhock to tolerate cold environmental conditions in order to evaluate future opportunities for unconventional agricultural practices. These novel insights will provide a better mechanistic understanding of cold tolerance in ornamental plants.

Materials and methods

Plant materials and growth conditions

The pot experiment was conducted in 2021–2022 at Ferdowsi University of Mashhad, Iran (36.3100° N, 59.5296° E). The experiment lasted seven months. In August 2021, healthy seeds of the *Alcea rosea* cultivar 'Nigra' were obtained from the Takii company, in Kyoto, Japan. Seeds were sown (6 cm deep) in the center of pots (24 cm wide and 30 cm long) filled with peat moss and coco coir (2:1, v:v) and grown under controlled conditions of a temperature of 23 ± 1 °C, a photoperiod of 16/8 h and a fluorescent light photon flux of 60 µmol m^{-2} s^{-1}. Soil moisture was maintained at ∼65%. The plants were irrigated with water per day. The pots were transplanted in the greenhouse for one month until October. Water soluble NPK fertilizer was applied once at the time of seedlings transplanting. The temperature was 20–21 °C during the day and 15 °C at night (relative humidity of ∼80%). The plants were allowed to grow in the cold frame for 2 months under natural sunlight, and temperature, acclimated to the growth condition. Plants were irrigated with well water during the adaptation period before treatment onset.

Se and Se application and cold stress

Si and Se treatments were imposed after 5 months of plants cultured under glasshouse and cold frame conditions. The experiment was conducted in a completely randomized design with four nutrition treatments and two temperatures with three replications. Factor one was nutrition, which was applied to the root medium as sodium selenite and sodium silicate (Sigma–Aldrich), and factor two was temperature, which was supplied as control (20 °C) and −4 °C. At the 6-leaf stage, four treatments were carried out by adding sodium sulfate and sodium silicate to 1/2 Hoagland solution, including 1) control, 1 mM sodium sulfate, 2) 1 mM sodium silicate 3), 0.03 mM sodium selenite, 4) 1 mM sodium silicate + 0.03 mM sodium selenite (the concentrations were selected based on a pretest; also sodium sulfate was used in the control solution to ensure the balance of sodium ions in the treatments). In February, the cold stress treatment was applied. By the previous result, the temperature of −4 °C was selected for freezing temperature (Oraee and Tehranifar 2022). Randomly selected plants were frozen in a termogradient. Seedlings were moved to a chamber with a controlled environment that was kept at 4 °C. After 30 min, the temperature was lowered to 2 °C, and the chamber temperature was decreased to −4 °C for 90 min. Five plants per treatment were placed and thawed overnight at 3 °C (Nezami, Bandara, and Gusta, 2012). After cold stress at the vegetative growth stage, the effect of optimized Se and Si in combination or separately on physio-biochemical and anatomical properties of leaves hollyhock (nutritional elements, photosynthesis parameters, phenol, water relative content, mucilage, and essential oil, proline, soluble sugar content, electrolyte leakage, malondialdehyde, antioxidant enzyme activities) were analyzed. Three weeks after the cold stress, the plant morphological index such as fresh and dry weights were measured.

Nutritional elements

Leaf samples were prepared for analysis. Each sample of cleaned green leaves (25.00 g) was dried in an oven (90 ± 5 °C). K^+ ion was determined using a flame photometer (atomic absorption

spectrophotometry; Chapman and Pratt 1961). Si and Se were determined using ICP-OES (Spectro Arcos: 76004555 plasma).

Photosynthesis parameters

First, one gram of the leaves was weighed, and then 5 ml of 80% acetone was added to a mortar. It was poured into a graduated cylinder, and the volume was made up to 10 mm with 80% acetone. By separating the impurities after centrifugation (4000 rpm for 10 min), the clear liquid containing chlorophyll was brought to a volume of 10 ml for the second time. Absorbance measurements were performed at 653 and 666 nm using a UV-2100 spectrophotometer (Ritchie 2008). After the application of cold stress, an assay of net photosynthetic rate (*Pn*) and stomatal conductance (*gs*) in the chamber was started in the youngest, fully developed leaf. A portable LCi photosynthesis system (ADC Bioscientific Ltd., Hoddesdon, England) was used to measure these traits. Stomata size and number were calculated as described by Nazdar et al. (2019). The leaf of the hollyhocks was dried, and the dried samples were mounted on a standard scanning electron microscope (SEM) with double-sided tape, coated with 25-nm gold-palladium using a Hummer II sputter coater, and examined at 20 kV using SEM (LEO1450VP). Endogenous ABA was assayed using an HPLC system as per the procedure given by Lang et al. (2019).

Relative water content essential oil and mucilage

Relative water content (RWC) was assayed on fresh leaf samples by following the method of Smart and Bingham (1974). Samples were mixed with 300 ml of distilled water, and essential oil content was determined by hydrodistillation for 3 h using a modified Clevenger apparatus (Shekari, Abbasi, and Mustafavi 2017). The mucilage was extracted using a hot extraction method. In summary, 2 g of dry samples were mixed with 10 mL of acidified distilled water (pH = 3.7). Then, 200 mL of distilled water was added and blended for 20 min. The waste products were separated using a Buchner funnel, and the remaining solution was centrifuged. Ethanol 96% (four times the volume of the solution) was added. The final solution was kept at 4 °C for 24 h to allow the mucilage to precipitate. The precipitate was separated by vacuum filtration using a Buchner funnel and then weighed after drying (Saeidi and Lorigooini 2017).

Electrolyte leakage and lipid peroxidation of membranes

The stability of the membrane was evaluated by measuring the leakage of leaf electrolytes (Sairam, Rao, and Srivastava, 2002). For this purpose, 100 mg of leaf samples were transferred to a container containing 20 ml of distilled water and kept at room temperature for 24 h. Then, the electrical conductivity of the distilled water was measured with the sample as the initial leakage. Secondary leakage was also calculated by measuring the electrical conductivity of the samples after heating at 100 °C for one hour. The amount of membrane peroxidation was measured by measuring the amount of malondialdehyde (MDA) and was determined by the Heath and Packer (1968) method. After the determination of fresh weight, the fresh plant tissue was homogenized in 20% trichloroacetic acid (TBA) and incubated in a hot water bath for 30 min, then the resulting mixture was immediately cooled in an ice bath. The mixture was immediately centrifuged at a speed of 3000 for 30 min. The absorbance of the supernatant was determined at 600 and 532 nm.

Quantification of antioxidant enzyme activities

Fresh leaves (100 mg) were pulverized in liquid nitrogen, and 1 mL of 0.1 M potassium phosphate buffer with Ph = 7.8, 2 mM EDTA was added for evaluation of enzyme activity. The insoluble was separated using a refrigerated centrifuge Sigma model K-18-3 at 12,000 rpm for 20 min at 4 °C, and the upper solution was used as a source for the extraction of the enzymes. One hundred µL of the upper solution was removed and placed in microtubes to determine the activity of ascorbate peroxidase, catalase, and superoxide dismutase at −80 °C. Ascorbate peroxidase activity (EC 1.11.1.11) was determined according to the method of Yamaguchi, Mori, and Nishimura (1995). The reaction mixture contained 50 mM potassium phosphate buffer (pH = 7), 1 mM hydrogen peroxide, and 0.5 mM ascorbate. The activity of ascorbate peroxidase was dependent on the decrease in absorbance at 290 nm, as ascorbate ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$) was oxidized for 3 min. Catalase activity (EC 1.11.1.6) was evaluated by the initial rate of disappearance of hydrogen peroxide according to the method of Velikova, Yordanov, and Edreva (2000). One mL of the catalase reaction solution contained ten mM potassium phosphate buffer (pH = 7) with 10 mL of extracted enzyme and 33 mM hydrogen peroxide. The decrease in absorbance at a wavelength of 240 nm in one minute and micromoles of hydrogen peroxide consumed per minute was defined as one unit of catalase ($\varepsilon = 40\ mM^{-1}\ cm^{-1}$). Superoxide dismutase (SOD) activity was analyzed by measuring photochemical inhibition by nitroblue tetrazolium (NBT) at 560 nm. A SOD unit was defined as the amount of enzyme amount that causes 50% inhibition of NBT reduction (Giannopolitis and Ries 1977).

Phenol, proline, and soluble sugar concentrations

The total phenolic concentration in the fresh leaf sample was determined by the Fullen-Cicalto method (Singleton and Rossi 1965). 100 mg of fresh leaf samples were homogenized with ethanol and stored at 4 °C for 14 h. Insoluble solids were separated by centrifugation at 3000 rpm for 5 min. Twenty µL of the extracted solution, one mL of double-distilled water, and 20 µL of Fullen-Cicalto reagent were added. After 5 min, 120 µL of sodium carbonate (20% by volume) was added and kept in the dark for 30 min. Total phenolic content was determined based on absorbance at a wavelength of 765 nm. The amount of proline in leaf tissue was measured according to the method of Bates, Waldran, and Teare (1973). A total of 100 mg of a fresh leaf sample was homogenized in 1 mL of sulfosalicylic acid (3%). Glacial acetic acid (200 µl) and 200 µl of ninhydrin reagent were added to 200 µL of the extraction solution. The reaction mixture was placed in a 100 °C water bath for 30 min. After cooling the reaction mixture, 600 µL of toluene was added. The proline concentration was read from the upper colored solution at 520 nm. The proline concentration was determined using the proline standard curve. Soluble leaf sugar was determined by the phenol sulfuric acid method (Dubois et al. 1956) and the glucose standard. Leaves (100 mg) were homogenized with ethanol; then samples were centrifuged for 5 min. The upper solution was mixed with chloroform and distilled water in volume ratios of two, two, and one, respectively. Then it was mixed with phenol in a volume ratio of two to one and finally brought to the indicated volume with 98% sulfuric acid. Finally, after 30 min in a hot water bath at a temperature of 100 °C, the absorbance at 480 nm was read.

Fresh and dry weight of vegetative organs

After 21 days, the fresh weight of the plant was determined using a digital scale, and the dry weight of the vegetative organs of the hollyhock was obtained through the dehydration of the sample in a thermo-ventilated oven at 80 °C.

Data analysis

The data were analyzed by analysis of variance analysis (ANOVA) and the means were compared using Tukey's multiple range tests at $p > .05$ (ANOVA SAS release 9.1; SAS Institute, Cary, NC, USA). Data were statistically analyzed for standard deviation and error using SAS release 9.1 (SAS Institute 2002).

Results

Effect of fertilizer and cold stress on nutritional elements

Si, Se, Si + Se significantly ($p \leq .05$) affected the selenium and silicon concentrations. The application of different fertilizers, specifically Si and Se, enhanced the uptake of nutrients, particularly potassium, in both cold-stress and non-cold-stress plants. No significant increase of selenium and silicon was found by cold stress. The application of Si + Se increased the selenium content in the leaves of plants by 188% compared to control plants. No significant difference was found between selenium accumulation in plants treated with Si and Si + Se. The application of Si or Si + Se increased the silicon in the leaves of the plants. The highest amount of silicon was observed in plants under Si + Se, so the silicon in plants treated with Si + Se was increased by 191% compared to control plants (Table 1). The concentration of K significantly ($p \leq 0.01$) is affected by the interaction of cold and fertilizer. Si + Se application considerably increased the K concentration by 49% and 55%, respectively, compared to control plants under normal and cold stress conditions. A significant decline in K concentration by 4.37% was observed under cold stress of control plants, while there were no differences between cold-stressed plants and control plants under Si, and Si + Se (Table 2).

Effect of fertilizer and cold stress on relative water content essential oil and mucilage

The relative water content (RWC) essential oil content and mucilage were higher in both Si-treated and Se-treated plants compared to the control group. There was no significant decrease or increase in relative water content (RWC) under cold stress. However, RWC significantly increased by 2.3% and 3.3% in plants treated with Si and Si + Se, respectively, compared to the control group. The essential oil was significantly affected by fertilizers. The essential oils of the plants were significantly increased by Si and Se, and the combination of Si and Se seems to have a more significant effect than the separate addition of Si and Se. Among the treatments, plants fed with Se + Si had a high essential oil content (3.3-fold) compared to the control (Table 1). The addition of Si resulted in a significant increase in the mucilage content of the hollyhock, with the value rising from 0.319 g to 0.548 in the Si + Se treatment. Both Se and Si applications led to a substantial increase in mucilage, but there was no significant difference between the two treatments.

Table 1. Mean comparison of main effects and significance of fertilizer and cold stress on Si, Se, stomata density, RWC, essential oil and mucilage of hollyhock.

Treatments	Si (μ mol g^{-1} DW)	Se (μ mol g^{-1} DW)	Stomatal density (num/ mm^2)	RWC (%)	Essential oil (%)	Mucilage (g)
Fertilizer						
Control	30.67b	36.5c	159.8b	78.05c	0.063d	0.218c
Si	615.67a	37.7c	160.1b	80.03ab	0.158c	0.319b
Se	34.83b	510b	160.7b	79.87b	0.182b	0.388b
Si + Se	614a	737a	164.8a	80.60a	0.208a	0.548a
Stress						
Non cold stress	350a	330.4a	161.3a	79.7a	0.153a	0.388a
Cold stress	296a	330.2a	161.4a	79.5a	0.152a	0.385a

Means followed by the same letter within each column shows no significant differences among treatments at 0.05 level by LSD. (Si: silicon, Se: selenium, RWC: Relative water stress; ns: not significant. $^{*}p < .05$, and $^{**}p < .01$).

Effect of fertilizer and cold stress on photosynthesis and ABA in leaves

From the results of ANOVA, Si, Se, Si + Se, and cold stress significantly ($p \leq .05$) affected the photosynthesis parameters. Under low-temperature conditions, chlorophyll levels, photosynthetic rate (*Pn*), stomatal conductance (*gs*), and stomatal size were significantly reduced in plants from the control group, as well as those treated with Si and Se. The Si, Se, or Si + Se addition increased the leaf's total chlorophyll of non-cold stressed hollyhock. Under cold stress and stress-free conditions, the Si + Se combination increased total chlorophyll by 44.2% and 20.6%, respectively, compared to the control. Si and Se application significantly improved the total chlorophyll by 41% and 35.6%, respectively, under stress-free conditions. Similarly, Si application substantially improved in total chlorophyll, 17.9%, under cold stress compared to the control. The highest amount of *Pn* (30.03–31.3 µmol m^{-2} s^{-1}) was seen in plants treated with Si + Se and Si under stress-free conditions, but cold stress reduced the amount of *Pn* by 34.6% compared to control plans. *Pn* expectedly decreased under low temperature conditions both in the presence and absence of Si and Se. Under cold stress, the control group exhibited the lowest *Pn* at a range of 17.6–18 µmol m^{-2} s^{-1}. Added Se and Si significantly reduced the *Pn* of stressed plants by 28% and 38%, respectively, compared to plants without cold stress. The highest *gs* (0.034 µmol m^{-2} s^{-1}) was observed in Si + Se treated stress-free plants. The stomatal conductance (*gs*) in stress-free and stressed plants treated with Si + Se decreased by 64.7% and 55.5%, respectively, compared to control plants. The application of Si, Se, or Si + Se resulted in a significant decrease in *gs*. In conditions without stress, there was no notable difference observed in *gs* between the Si or Se treatments (Table 2). While there was no significant difference in stomatal density under cold stress, it was influenced by the application of Si, Se, and Si + Se. The only significant differences ($p < 0.01$) were detected between the stomatal density in Si + Se application, and it significantly increased (by 3.14%) compared to control plants (Table 1). Figure 1 depicts variations in stomatal size and density of hollyhock leaves when cultivated with different fertilizers. Significantly larger stomatal size was observed in the leaf base of plants treated with Si + Se compared to the control group, both under cold and non-cold stress conditions. The arrangement of these larger stomata was more intricate in the Si + Se treatment. The size of hollyhock stomata and ABA were significantly ($p < .01$) influenced by both fertilizer treatment and cold stress. In the presence of Si + Se, the stomatal size of plants exposed to cold stress increased by 65.2%, while non-cold stressed plants showed a 100% increase compared to the control group. Additionally, the application of Se and Si led to significant improvements in stomatal size by 7.5% and 8.4%, respectively, under normal conditions (Table 2). The Si + Se treatments resulted in ABA content that was 51.1% and 18.4% higher than the control group in non-stressed and cold-stressed plants, respectively. Cold stress exposure caused a significant increase in the ABA content of plants treated with Si and Se by 13.5% and 6.65% in contrast to the control (Table 2).

Table 2. Effect of Si, Se and cold stress on potassium, chlorophyll, *Pn*, *gs*, stomata size, ABA content in daisy leaves.

Fertilizer	Stress	K^+ (µ mol g^{-1} DW)	Chlorohyll (mg g^{-1} FW)	*Pn* (µmol m^{-2} s^{-1})	*gs* (µmol m^{-2} s^{-1})	Stomatal size (µm²)	ABA (ng g^{-1} DW)
	Non cold stress						
Control		640 ± 5.77c	8.034 ± 0.018b	24.5 ± 0.577b	0.034 ± 0.002a	213 ± 1.63c	1.31 ± 0.001e
Si		938 ± 4.32a	10.9 ± 0.507a	30.3 ± 0.162a	0.022 ± 0.001b	229 ± 2.31b	1.37 ± 0.882e
Se		732 ± 3.05b	11.4 ± 0.067a	25 ± 0.577b	0.022 ± 0.001b	231 ± 0.647b	1.42 ± 1.20e
Si + Se		955 ± 2.96a	11.6 ± 0.007a	31.3 ± 0.580a	0.012 ± 0.001c	352 ± 1.73a	1.98 ± 0.577d
	Cold stress						
Control		612 ± 0.88d	6.5 ± 0.058d	17.6 ± 0.565d	0.009 ± 0.002 cd	173 ± 1.73e	4.06 ± 0.33c
Si		939 ± 9.33a	6.8 ± 0.001 cd	18.1 ± 0.001d	0.008 ± 0.002d	197 ± 1.15d	4.61 ± 1.20ab
Se		727 ± 4.67b	7.67 ± 0.033bc	18.8 ± 0.484 cd	0.008 ± 0.002d	220 ± 1.15c	4.33 ± 1.33bc
Si + Se		952 ± 5.14a	7.84 ± 0.009b	20.5 ± 0.392c	0.004 ± 0.001e	347 ± 1.15a	4.81 ± 1.20a

Means within each column followed by the same letter are not statistically different at ≤0.05 by Least Significant Difference test. (*Pn*; photosynthesis rate, *gs*; stomatal conductance, ABA; abscisic acid).

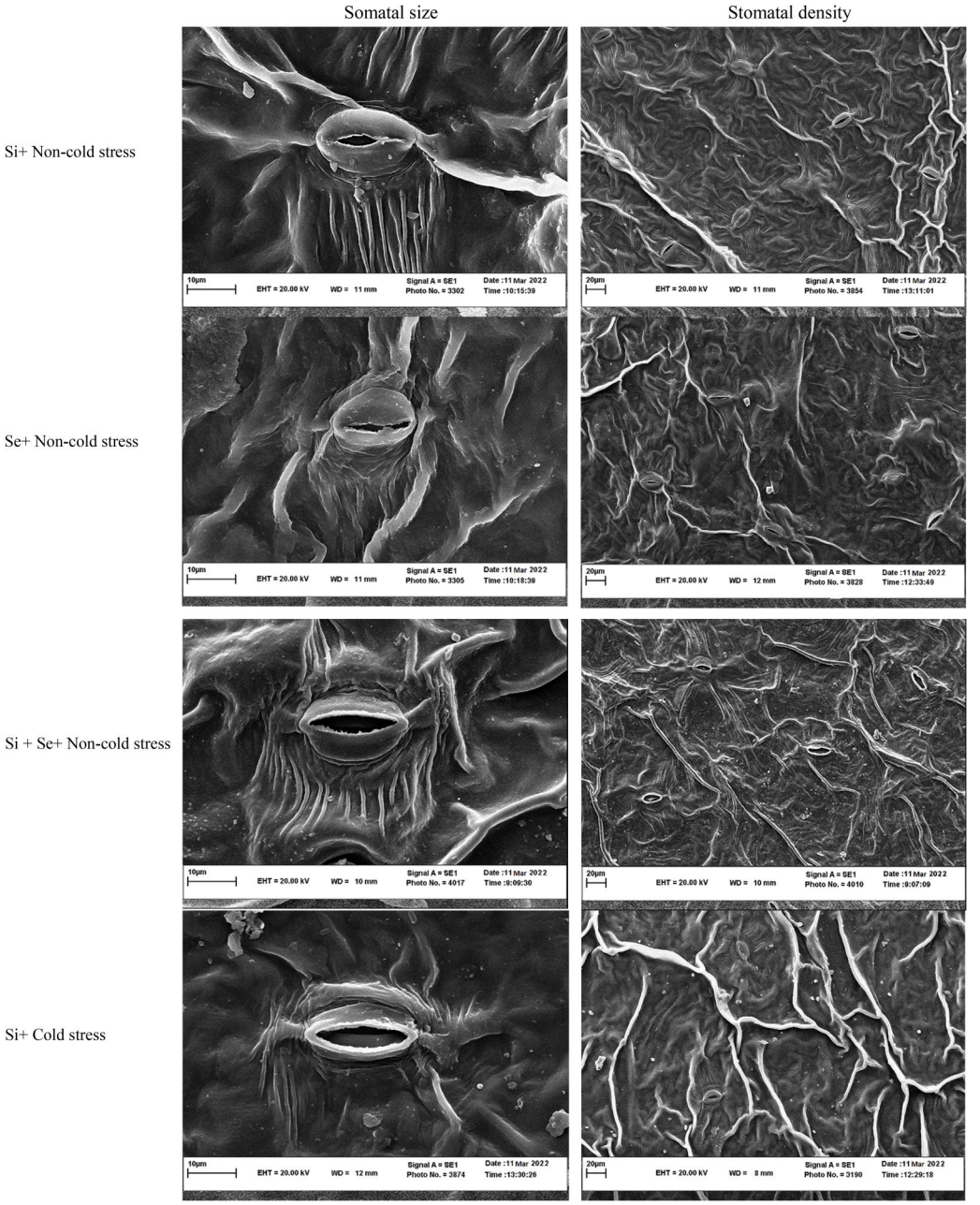

Figure 1. Stomata size and stomatal density in the leaf of non cold stressed and cold-stressed hollyhock fed with or without Si and Se (SEM images of hollyhocks at 5000 and 1000× magnification).

Effect of fertilizer and cold stress on phenol, proline, and water-soluble sugar

The results indicate that Si, Se, Si + Se, and cold stress had a significant ($p < .01$) effect on the phenol content. The interactions between fertilizer treatments and cold stress had a significant effect on the levels of phenols, proline, and water-soluble sugars. This indicates that the beneficial effects of Si and Se were primarily observed under low-temperature conditions. Cold stress resulted in a 28.1% increase in phenol content in the control plants. Under cold stress conditions,

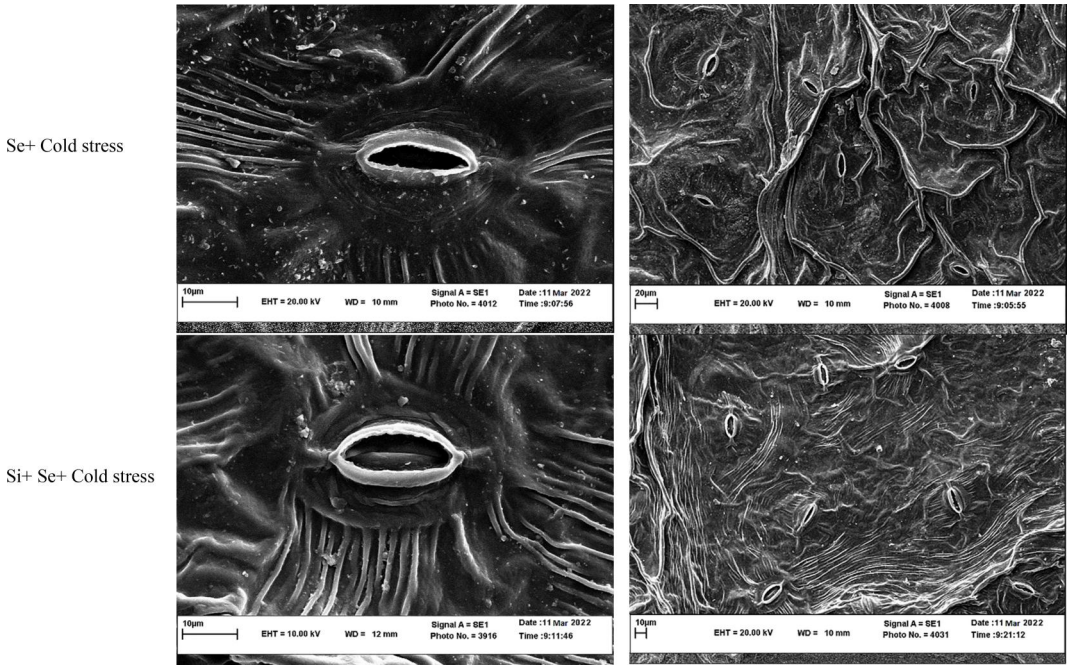

Figure 1. Continued

the application of Se, Si, and Si + Se increased the phenol content by 23.8%, 23.6%, and 48.4%, respectively, compared to stress-free plants (Figure 2(a)). Under both normal and fertilizer conditions, the proline content was significantly higher in plants subjected to cold stress compared to non-cold-stress conditions. Si, Se, Si + Se, and cold stress had a significant effect on proline and water-soluble sugar content in leaves. Specifically, the combination of Si + Se resulted in a 36.5% increase in proline content in stress-free plants and a 19.3% increase in stressed plants compared to the control group. Additionally, under stress conditions, Se and Si caused a 36.3% and 29.7% increase in proline content, respectively, compared to stress-free conditions (Figure 2(b)). In both non-cold and cold stress conditions, all fertilizers led to an increase in the amount of water-soluble sugar. The control plants experienced a 1.2-fold increase in water-soluble sugars under cold stress compared to stress-free conditions. When silicon (Si) and selenium (Se) were applied, there was a significant improvement in water-soluble sugars for both cold and non-cold stressed plants. Compared to the control group without Si or Se application, the water-soluble sugars increased by 25% and 23.8%, respectively, for cold and non-cold stressed plants with Si + Se application. Additionally, Se and Si application resulted in a 6.58% and 11% improvement in water-soluble sugars under cold stress compared to the control group (Figure 2(c)).

Effect of fertilizer and cold stress on electrolyte leakage and MDA

Electrolyte leakage and MDA significantly were affected by Si, Se, Si + Se, and cold stress. Under both normal and cold temperature conditions, the Si + Se treatment resulted in significantly lower levels of electrolyte leakage and malondialdehyde (MDA) compared to the treatment without Si. Cold stress resulted in a 44.1% increase in electrolyte leakage compared to the control group. However, the application of Si, Se, and Si + Se reduced electrolyte loss in both stressful and non-stressful conditions. Under stressful conditions, Se, Si, and Si + Se reduced electrolyte loss by 53.8%, 118%, and 116%, respectively, compared to stress-free conditions (Figure 2(d)). The

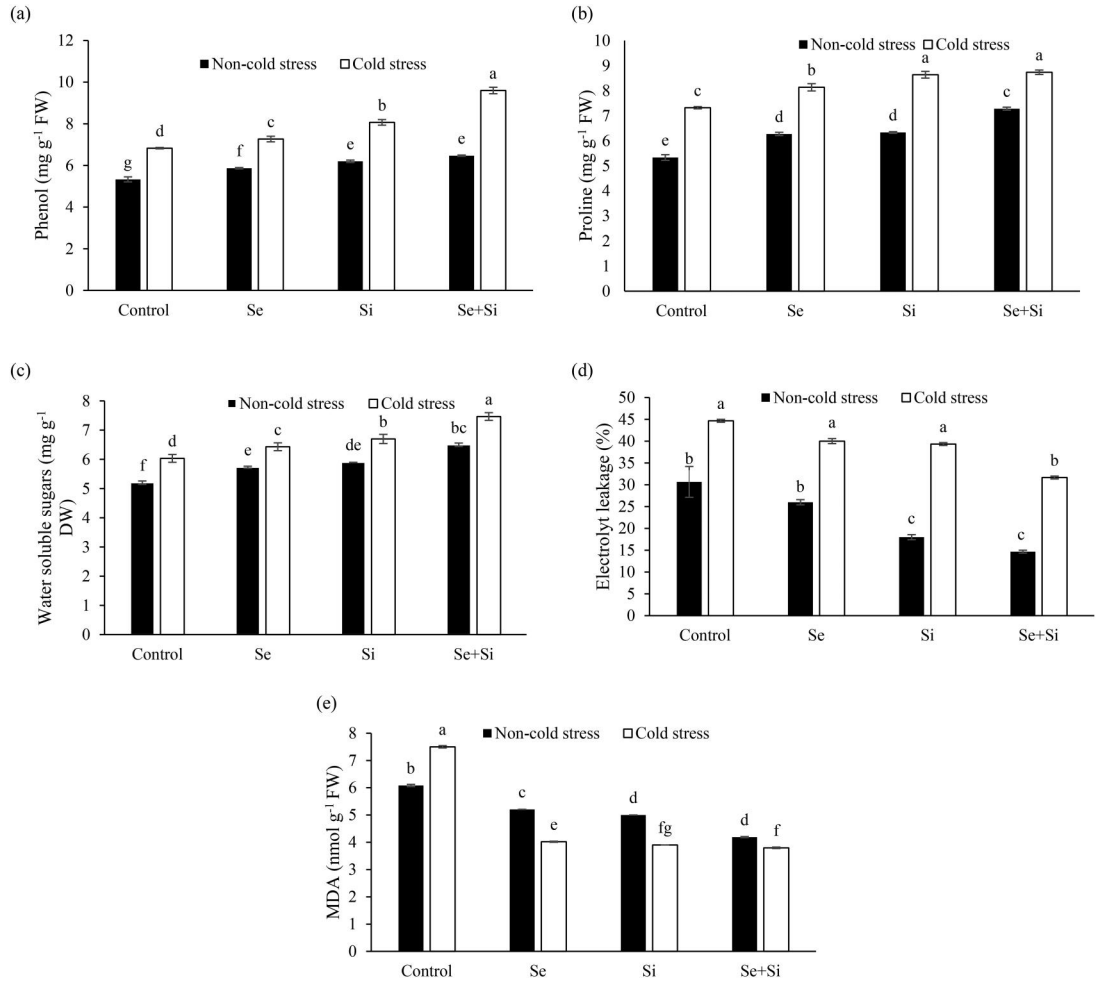

Figure 2. Electrolyte leakage (a), MDA (b), phenol (c), proline (d), and water soluble sugars (e) in the leaf of control and cold-stressed hollyhock plants fed with or without Si and Se. Values are mean ± SE based three replications ($n = 3$). No Si and Se treatments were in the control. Treatments with the same letter were not significantly different ($p \leq .05$) according to tukey's multiple range test.

separate application of Si and Se did not have a significant effect on MDA content under cold stress. However, MDA content was significantly affected by Si, Se, Si + Se, and cold stress. The control plants subjected to cold stress had the highest MDA content (7.5 nmol g^{-1} FW). Si, Se, and Si + Se treatments decreased MDA content under both stress-free and cold-stress conditions. Specifically, the combination of Si and Se reduced MDA content by 31% under stress-free conditions and 49.4% under stress conditions compared to the control group. Additionally, under stress-free conditions, the addition of Se and Si to the solution led to a significant reduction in MDA content by 14.5% and 17.8%, respectively, compared to the control group (Figure 2(e)).

Effect of fertilizer and cold stress on antioxidant enzyme activities

Si, Se, and Si + Se supply also positively influenced the antioxidant activities, including APX, CAT, and SOD in the leaves in cold and non-cold stress conditions. Si and Se treatments induced significant alterations in the activities of antioxidant enzymes, leading to enhanced cold tolerance in the plants. This was achieved by increasing the levels of ascorbate peroxidase (APX), catalase

Table 3. Effect of Si, Se and cold stress on APX, CAT, SOD, and fresh and dry weights of daisy leaves.

Fertilizer	Stress	APX (μ mol min^{-1} g^{-1} FW)	CAT (μ mol min^{-1} g^{-1} FW)	SOD (U g^{-1} FW)	Fresh weight (g)	Dry weight (g)
	Non cold stress					
Control		16.1 ± 0.289f	21.5 ± 0.288f	100 ± 5.77d	44.12 ± 0.007c	41.1 ± 0.577e
Si		23.3 ± 0.440e	28.3 ± 0.440de	108 ± 3.33 cd	48.10 ± 0.033b	66.4 ± 0.203b
Se		28.1 ± 0.577d	30.1 ± 0.577 cd	116 ± 3.05c	48.97 ± 0.033b	50.1 ± 0.58d
Si + Se		31.7 ± 0.400c	30.3 ± 0.333c	120 ± 2.88bc	56.70 ± 0.057a	71.1 ± 0.067a
	Cold stress					
Control		23.1 ± 0.33e	28.1 ± 0.577e	111 ± 1.33 cd	39.99 ± 0.033d	38.3 ± 0.067f
Si		30.5 ± 0.484c	34.3 ± 0.333b	123 ± 3.33bc	43.20 ± 0.0333c	63.4 ± 0.60d
Se		37.3 ± 0.440b	36.2 ± 0.167b	132 ± 1.20b	4.390 ± 0.067 cd	48.3 ± 0.333e
Si + Se		41.4 ± 0.464a	39.8 ± 0.167a	151 ± 0.67a	47.92 ± 0.067b	66.3 ± 0.218b

Means within each column followed by the same letter are not statistically different at ≤0.05 by Least Significant Difference test. (APX; ascorbate peroxidase, CAT; catalase, SOD; superoxide dismutase).

(CAT), and superoxide dismutase (SOD) under cold conditions. The Si + Se treatment showed the highest increase in APX activity, with enhancements of 100% and 83% observed in stress-free and cold-stress conditions, respectively. The addition of Se also had a positive impact on APX activity in cold-stressed plants, reaching a value of 37.3 µmol min^{-1} g^{-1} FW. In comparison, plants treated with Si alone exhibited a 75% and 62% increase in APX activity compared to control plants under cold stress and stress-free conditions. The Si + Se treatment resulted in significantly higher activities of CAT and SOD compared to the -Si treatment in both normal and cold temperature conditions. The activities of CAT, however, was significantly higher under cold stress. In terms of cold stress tolerance, the Si + Se treatments led to a significant 42.8% increase in maximum CAT activity compared to the control group. The CAT activity were 39.5% and 28.5%, respectively, in Si treatments under non- and cold-stressed condition than control. Under stress-free and cold conditions, the activity of SOD was increased by 36% and 20% respectively, in plants treated with Si + Se compared to the control group. The application of Si alone improved SOD activity by 16% under cold conditions and 20% under stress-free conditions compared to the control group. Although the addition of Si and Se increased SOD activity under stressful conditions, there was no significant difference observed between plants treated with Si and Se (Table 3).

Effect of fertilizer and cold stress on fresh and dry weights

Plant growth was significantly inhibited under cold stress compared to normal conditions. Increasing cold stress decreased fresh and dry weights by 9.36% and 6.51%, respectively, compared to the control plant. However, the inhibition of plant development by cold stress was mitigated by Si, Se, and Si + Se application. Under cold stress, the fresh weight of plants was significantly greater in the Si + Se treatment compared to the Si and Se treatments. The Si + Se application resulted in the highest fresh weight under non-cold conditions, while the lowest fresh weight was observed in cold-stressed plants without any fertilizer. Under stress and non-stress conditions, added Se and Si individually or in combination increased plant dry weight, with the combination of Se and Si causing the highest dry weight increase under non-stress (73.3%) and stress (7.8%) conditions compared to control plants. Dry weight under cold and non-cold stress conditions was not affected by Se application, but these treatments increased the dry weight of the hollyhock compared to the control (Table 3).

Discussion

Silicon and selenium play vital roles in enhancing crop growth and bolstering crop resilience against both biotic and abiotic stresses. It is a key factor in promoting plant development and

fortifying plants against various challenges, including diseases, pests, and adverse environmental conditions. Previous studies have shown significant differences in physiological traits in plant leaves after silicon and selenium addition. In this work, Si and Se additions improved all assessed biochemical and physiological parameters. The amount of Si, Se, and K in the leaves increased significantly compared to control plants. The accumulation of Se and Si in hollyhock leaves was proportional to the concentration of Se and Si. The contents of Se and Si in the leaves of the control seedlings were 615 and 510 mg g^{-1} dry weight. In the leaves grown in the nutrient solution with the addition of Si + Se, the Si and Se contents were 737 and 614 mg g^{-1} dry weight. The results of previous work showed that adding sodium silicate and sodium selenite to a nutrient solution led to an accumulation of Se, Si, and K in plants (Hawrylak-Nowak 2013; Xu et al. 2021). In addition to Si and Se, redistribution of K was also examined because it is well known as an indicator of nutrient leaching in response to tissue and membrane integrity impairment (Sardans and Peñuelas 2021) and its function in protection from cold stress (Donderalp and Dursun 2022). At the end of the cold stress period, the untreated control seedlings had lost 4.37% of their K leaf, while the K concentration in the Si and Se-treated leaves had not changed significantly. As shown in Table 2, Si + Se addition caused the increase in leaf potassium content of cold and non-cold stressed plants. Silicon and selenium have been shown to enhance plant tolerance to cold stress by improving potassium uptake and transportation in plants. They help maintain potassium homeostasis and regulate its distribution within plant tissues. Silicon application can increase the expression of potassium transporters, facilitating potassium uptake from the soil and its movement to the shoot tissues. Selenium also enhances the activities of enzymes involved in potassium uptake and utilization, promoting better potassium absorption and utilization by plants under cold stress conditions (Wang et al. 2022). Similar effects of Si and Si were observed under non-cold conditions. This is in full agreement with the recent report which showed that the supply of Si and Se significantly increased potassium in plants (Pazurkiewicz-Kocot, Galas, and Kita 2003; Shekari, Abbasi, and Mustafavi 2017).

In this study, both Si and Se treatments were found to enhance photosynthesis parameters. However, it was observed that these traits showed greater improvement under non-cold conditions. The increasing effects of selenium and silicon on chlorophyll, and photosynthesis rate (*Pn*) played a role in enhancing cold resistance by influencing relative water content (RWC) (Tables 1 and 2). The findings of this study supported previous knowledge in this field. Si and Se treatments decreased *gs* in the present study. According to Meena et al. (2014), it was proposed that Si can be deposited in the form of silica gel within the epidermal cell walls of plants, which helps to reduce water loss through transpiration. Many researchers have suggested that the formation of a cuticle-silica double layer on the epidermal tissue of the leaf is responsible for the reduction in transpiration of the silicone-treated leaf (Agarie et al. 1998). This results in lower water loss and increased relative water content, as reported by Taha et al. (2021). Different studies have provided varying explanations for how silicon inhibits water loss. Some researchers have suggested that silicon reduces stomatal conductance (Vandegeer et al. 2021), while others have proposed that it decreases the accumulation of H_2O_2 and stimulates the expression of aquaporin genes, enabling roots to uptake more water during stress conditions (Zargar et al. 2019).

The cold stress had a negative impact on plant development and caused significant leaf damage. Exposure to low temperatures commonly reduces important physiological parameters in hollyhocks such as chlorophyll, *gs*, *Pn*, and stomatal size. Stomatal closure is one of the initial responses of plants to cold stress, which helps them tolerate low temperatures and reduce CO_2 levels. The decrease in CO_2 fixation can disrupt the balance between light absorption and utilization, and also alter the photochemistry of chloroplasts, resulting in a reduced photosynthetic rate. This imbalance between electron release and acceptance in the photosystems leads to an accumulation of reactive oxygen species (ROS) (Dreyer and Dietz 2018). Partelli et al. (2009) demonstrated that an increase in reactive oxygen species (ROS) leads to lipid peroxidation and

membrane damage, resulting in chlorophyll loss and degradation of leaf tissue in coffee plants exposed to low temperatures. Inhibition of chlorophyll synthesis, acceleration of degradation, and oxidative stress caused by cold stress harm chlorophyll content (Zhang et al. 2022). Adding Si and, or Se decreased the negative impact of cold stress on chlorophyll (Table 2). This finding is consistent with a recent report that demonstrated the significant enhancement of cold tolerance through the supply of Si and Se, which increased photosynthesis parameters. Moreover, previous results showed that the chlorophyll content promoted by Si and Se was due to enhanced biosynthesis of new chlorophylls and protection of existing chlorophyll from oxidative stress (Dong et al. 2013; Xu, Guo, and Liu 2022). Plant growth is mainly dependent on photosynthesis. We observed increased *Pn* in non-cold-stressed and cold-stressed plants exposed to Si + Se treatment (Table 2). Higher photosynthetic rates following Se treatment have also been reported for maize exposed to low temperatures (Elsheery et al. 2020), but the mechanisms responsible, particularly those independent of stomatal conductance, remain largely unexplored. A large amount of data on the recovery of Si and Se in plant growth and photosynthesis makes it logical that Si and, or Se keep photosynthesis high under stress. Si and Se reduce the adverse effects of cold by maintaining *Pn*, membrane permeability, and chlorophyll content (Cao et al. 2022; Elsheery et al. 2020). Previous findings show that Si and, or Se have a positive effect on chlorophyll and photosynthesis because they increase high K^+ uptake under stress conditions (Shekari, Abbasi, and Mustafavi 2017). Si and Se treatments showed similar effects on potassium (K) content, especially under cold conditions. There was a significant difference in K values between the Si + Se treatment and the control group.

The application of Si + Se to plants resulted in increased responsiveness of guard cells to ABA signaling, leading to more pronounced stomatal closure. This response is attributed to a higher efflux of potassium ions across the guard cells (Table 2). The data demonstrate that plants (Si + Se) exhibit a greater degree of stomatal closure with an increase in ABA concentration compared to plants without Si and Se treatment. This enhanced stomatal kinetics can potentially improve the efficiency of water usage in leaves by promoting CO_2 uptake while reducing unnecessary water loss, as discussed in the study by Vandegeer et al. (2020). The efflux of potassium ions from guard cells plays a crucial role in the signaling process of ABA. When there is an increase in endogenous ABA levels during cold stress, the first response is the activation of anion channels that facilitate the efflux of anions. This efflux leads to the depolarization of the plasma membrane of guard cells, subsequently activating outward potassium channels. As a result, there is a flow of potassium ions out of the guard cells, leading to a loss of turgor and subsequent closure of stomata (Chen et al. 2017). The findings are in agreement with those obtained by other authors.

Plants subjected to cold stress did not show significant differences in essential oil and mucilage production. However, the application of Si and Se resulted in a 3.3-fold and 1.8-fold increase in these indices, respectively. It is worth noting that the impact of Si or Se on leaf mucilage has not been thoroughly investigated. Only one study has examined the effect of Si on mucilage production in root border cells exposed to aluminum toxicity (Xiao and Liang, 2022). The mechanisms underlying the effects of Si and Se on essential oil and mucilage synthesis in plants have not been extensively explored, but several potential mechanisms can be considered. The mechanism of Si and Se on essential oil under different stress involves the regulation of gene expression, activation of antioxidant systems, stimulation of stress-response pathways, and enhancement of nutrient uptake and assimilation (Azimi et al. 2021; Shekari, Abbasi, and Mustafavi 2017). There is increasing evidence in the literature that feeding plants with Se and Si increase their biological value (Golubkina et al. 2022). Amiripour et al. (2021) showed that the administration of silicon could increases the essential oil of coriander under saline conditions. When Si was supplied, various biochemical responses such as an increase in essential oil in aromatic plants such as Damask Rose was reported, which could be due to the effect of Se on CO_2 assimilation, rubisco activity,

and photosynthetic pigment content (Farahani et al. 2021). The Si and Se supply in hollyhocks supports our hypothesis that supplementation can be used as an effective plant biofortification strategy to increase essential oil and mucilage content in areas with low Si and Si supply.

The application of silicon and selenium treatments had beneficial effects on the levels of electrolyte leakage and malondialdehyde (MDA) in seedlings, both under cold stress and normal conditions throughout the experiment. Although there was an increase in electrolyte leakage and MDA under both conditions, the levels were higher under cold stress compared to the control. Notably, the combined application of silicon and selenium (Si + Se) demonstrated superior results in reducing electrolyte leakage and MDA levels. Several researchers have supported these findings with similar results, highlighting the potential of Si and Se priming to mitigate oxidative damage by reducing electrolyte leakage and inhibiting lipid peroxidation under unfavorable conditions. As expected, hollyhock plants exhibited higher MDA concentrations under cold stress, indicating cell membrane damage (Figures 2(a and b)). Cold stress in plants can negatively affect the electron transfer chain in mitochondria and chloroplasts and disrupt entirely the regulatory process through imbalance (Zhang et al. 2021). Molecular oxygen (O_2) acts as an electron acceptor, leading to the overproduction of ROS, including OH^- and O^{2-}. Superoxide radicals and ROS are highly oxidizing compounds that can damage membrane and plasma systems (Fujii, Homma, and Osaki 2022). Lipid peroxidation by oxidation of unsaturated fatty acids leads to the accumulation of oxygen free radicals in stressed plant cells, ultimately resulting in membrane damage (Ayala, Muñoz, and Argüelles 2014). Plasma membrane permeability shows that Si and Se-treated and non-Si-treated plants show different responses. Pretreatment of plants with silicon significantly improved the effects of cold on membrane integrity and lipid peroxidation (Liang et al. 2008). According to Shi's report (2016), the administration of Si increases plasma membrane activity by reducing electrolyte loss, inhibiting ROS overproduction, and thus reducing lipid peroxidation of the membrane and improving the integrity of the plasma membrane. The role of Se reduction in cold-induced cell damage has been demonstrated. Abbas (2012) and Nandagopal et al. (2022) showed that applying Se to plants under cold-induced a significant reduction in MDA. Our results show that cold-induced cell damage is reduced by Si and Se, which have been shown to reduce electrolyte and malondialdehyde loss and increase antioxidant activity. These healing responses to cellular damage may be partially related to the enhanced antioxidant response and activity of ROS inhibitory enzymes by protecting cells from free radicals (Tang et al. 2015).

Si + Se application resulted in the highest levels of phenol, proline, and water-soluble sugars under both cold stress and normal conditions. While the accumulation of these compounds was observed under normal conditions, it was not as significant as under cold stress. Based on previous findings, Si and Se treatments provided more osmolytes to the seedlings under cold stress, which could potentially benefit hollyhocks in coping with cold stress. Si and Se have been shown to increase the concentration of phenolic compounds in various plant species under stress conditions. For instance, research conducted on barley (Vega et al. 2020), spinach (Saffaryazdi et al. 2012), and *Lepidium sativum* (Elguera et al. 2013) demonstrated that Si and Se treatments led to an increase in phenolic compounds. The enhanced accumulation of phenol compounds through Si and Se treatments suggests that these elements can effectively enhance a plant's ability to cope with cold stress by boosting its defense mechanisms. It is worth noting that Si treatments are likely to increase glucose, an essential substance in metabolic pathways. Additionally, studies have shown that both selenium (Walaa et al. 2010) and silicon (Hajiboland et al. 2018) treatments significantly increased the activity of Phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO) in plants.

In this study, the highest proline concentration was obtained from Si + Se under cold; the second highest one was obtained from Si application under cold stress as well. The lowest MDA was also obtained Si + Se under cold stress. Increasing MDA triggered proline metabolism. MDA level obtained from Si + Se sampling under cold decreased probably because of the increasing

proline concentration. Stress causes ROS accumulation in plants and results in MDA production. MDA is an indicator of oxidative damage and lipid peroxidation in higher plants under stress (Tang et al. 2015). An increase in osmolytes in plants such as pansy (Oraee et al. 2018) and *Calendula officinalis* (Jan et al. 2018) under cold stress has been reported previously. Carbohydrates and proline also help reduce ice nucleation in the apoplast and block ice propagation from the apoplast to intracellular spaces, preventing lethal intracellular freezing damage (Liu 2015). Proline affects several aspects of physiological changes in plants: Protection of osmotic potential, scavenging of free radicals and ROS, protection of molecules from denaturation, and regulation of cell pH—all of which contribute to the role of proline in cold stress (Sarkar, Bhowmik, and Shetty 2009). In the present study, the application of Si and Se increased the proline and soluble sugar content of leaves. The mechanism of Si and Se on proline and carbohydrates in plants under different stress involves several processes including, regulation of gene expression, stimulation of stress-responsive pathways, and enhancement of nutrient uptake and assimilation (Manivannan et al. 2016; Zhu et al. 2020). Si and Se treatments have been shown to regulate the expression of genes involved in the synthesis and accumulation of proline and carbohydrates. This regulation leads to an increase in the production of these compounds, which serve as osmoprotectants and energy sources during cold stress (Kavi Kishor and Sreenivasulu 2014). Si activaties specific stress-responsive signaling pathways in plants, such as mitogen-activated protein kinase (MAPK) cascades. These pathways regulate the expression of genes involved in proline and carbohydrate metabolism, leading to increased synthesis and accumulation of these compounds under different stress (Kumar et al. 2023). Si and Se treatments improve the uptake and assimilation of essential nutrients, including nitrogen and carbon, which are required for the synthesis of proline and carbohydrates. This enhanced nutrient availability promotes the accumulation of proline and carbohydrates in plants under cold stress (Wang et al. 2022). It appears that these inorganic elements increase the production of soluble sugars by promoting photosynthesis, which is probably effective as osmolytes in maintaining water balance and RWC (Table 1), in addition to promoting growth (Zahedi et al. 2020). The content of carbohydrates and proline in leaf cells is related to the photosynthetic rate, the partitioning of photosynthesis between starch and sucrose, and the rate of phloem loading (Lemoine et al. 2013). In this work, the accumulation of carbohydrates and proline was associated with a higher photosynthetic rate in Si + Se cold-stressed plants (Table 2). These results are in agreement with previous reports regarding the beneficial effects of Si and Se on the carbohydrate and proline seashore paspalum turfgrass (He et al. 2010) and tea under cold stress (Liu et al. 2021) conditions.

In cold temperatures, the hollyhock plant experienced a noticeable increase in MDA content, indicating the presence of reactive oxygen species (ROS) and potential damage to plant cells. However, applying Si and Se significantly reduced the MDA content, thereby protecting the plant's membranes from lipid peroxidation caused by chilling stress. This reduction in MDA content was linked to the enhanced activity of APX and the sustained activity of SOD and CAT after Si + Se application. Both low temperature and Si increased the activity of SOD and CAT in the leaf hollyhock, with low temperature having a stronger effect than Si and Se alone. The highest activities of CAT and SOD were observed in plants treated with Si + Se under cold stress, with no significant difference between Si and Se individually. The increased activity of these antioxidant enzymes, facilitated by Si or Se, is likely one of the main mechanisms that enable hollyhock plants to tolerate both nonfreezing and freezing temperatures. In a study conducted by Liu et al. (2021), it was found that adding external Se increased the activities of CAT, POD, and SOD under cold stress in tea (*Camellia sinensis*) plants. This, in turn, improved the plants' tolerance to cold temperatures and resulted in an increase in the ratio of unsaturated to saturated fatty acids, which was induced by cold stress. Similarly, Joudmand and Hajoboland et al. (2018) and Amiripour et al. (2021) discovered that applying external Si enhanced the stress tolerance of barley and coriander plants to cold and salt stress. This enhancement was attributed to the

improvement of antioxidant mechanisms, which helped reduce membrane damage caused by the cold stress. Additionally, Gong et al. (2005) found that applying silicate to wheat plants increased the ratios of unsaturated fatty acids in glycolipids and phospholipids, as well as the overall amount of membrane lipids in the plants. The presence of higher levels of membrane lipids in hollyhock plants may help to protect against the burst of reactive oxygen species caused by chilling stress, providing greater stability to the cell membranes. This increased membrane stability is supported by the slower increase in relative electrolyte leakage observed in the plants. These findings suggest that the application of Si and Se may be crucial in maintaining the integrity, stability, and functionality of the membranes in hollyhock plants. By enhancing antioxidative defenses, Si and Se help to reduce membrane permeability and mitigate the negative effects of chilling stress on the plants.

In this study, the combination of Si and Se treatments resulted in high values for all growth attributes in hollyhock plants under both normal and cold stress conditions. The application of Si and Se increased the growth attributes of hollyhock plants in both conditions. These findings suggest that the use of Si, Se, or a combination of both can help prevent excessive weight loss in hollyhocks under cold stress. One possible explanation for this effect is that Si and Se treatments restrict the stomatal conductance (*gs*) under cold stress, leading to an increased rate of photosynthesis (Gao et al. 2006; Vandegeer et al. 2020). This, in turn, results in higher levels of chlorophyll and relative water content (RWC). Furthermore, Si and Se treatments were also found to be effective in promoting growth under non-cold conditions, possibly due to similar mechanisms as mentioned earlier. The positive impact of Si on the growth of various plant species has been well-established. The use of silicon increased the height, stem diameter, leaf area, and plant weight (Salim et al. 2021). The application of selenium also increased the root weight and fresh and dry weight of shoots (Saffaryazdi et al. 2012). Additionally, Si and Se seem to have a crucial role in fresh and dry weights mitigation, as there is evidence that Si helps to control the photosynthesis rate. Photosynthesis is a metabolic process in which plants synthesize organic compounds and store energy and is a significant indicator of plant growth status. In our study, we observed that the combined treatment of Si and Se had a more beneficial impact compared to individual treatments under stress conditions. This could be attributed to the influence of Si and Se on the net rate of photosynthesis and their ability to enhance water use efficiency (Cao et al. 2022). These findings align with previous research indicating a strong correlation between photosynthesis rate and cold tolerance in plants (Joudmand and Hajiboland 2019; Liu et al. 2021).

Conclusion

Cold stress is a significant environmental challenge that requires effective mitigation methods. In our study, we investigated the use of silicon and selenium to alleviate the adverse effects of cold stress on the physiological and morphological traits of hollyhock plants. Direct exposure to cold stress resulted in oxidative damage, as evidenced by elevated levels of MDA and electrolyte leakage. This led to a decrease in chlorophyll content, photosynthetic rate (*Pn*), and stomatal conductance (*gs*), ultimately resulting in reduced fresh and dry weights of the plants. The application of Si + Se played a crucial role in enhancing cold stress tolerance in hollyhock plants. This was achieved through increased uptake of essential nutrients (K, Si, and Se), synthesis of secondary metabolites (phenol), osmoregulation (proline and water-soluble sugars), optimization of photosynthetic parameters (chlorophyll, *Pn*, and stomatal size), and activation of antioxidant enzyme activities (APX, CAT, and SOD). Both silicon and selenium supplementation improved cold tolerance in hollyhock plants. However, silicon had a more pronounced effect by significantly improving the dry weight of the plants during the recovery period. This improvement was attributed to the positive impact of silicon on K content, proline, phenol, and water-soluble sugars. The results also showed that the maximal to minimal values of essential oil content, relative water content

(RWC), and stomatal density depended on the treatment: Si + Se > Si = Se. This suggests that the combined application of silicon and selenium yielded the best results. The addition of Si and Se in hollyhock plants supported our hypothesis that supplementation can serve as an effective strategy for plant biofortification, particularly in areas where silicon and selenium availability is limited. Further studies are needed to investigate the long-term effects of different silicon and selenium concentrations on cold stress responses.

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