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Phenylalanine, Cysteine, and Sodium Selenate Alleviate Chilling Injury in Cape Gooseberry (*Physalis peruviana* L.) Seedlings by Enhancing Antioxidant Activities and Membrane Stability

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Abstract: Low temperature is a major environmental factor that negatively affects the growth and productivity of plants, such as the tropical fruit Cape gooseberry (*Physalis peruviana* L.), which is susceptible to cold stress. Therefore, to investigate the effect of the amino acid L-phenylalanine (Phe), L-cysteine (Cys), or sodium selenite (Se) on enhancing antioxidant activities, experiments were conducted on the phenolic compounds, proline content, and membrane stability of Cape gooseberry seedlings under low-temperature stress. The seedlings were exposed for 48 h to a low temperature (4 °C) followed by 24 h of optimal growth conditions. In seedlings treated with Se, we found a high relative water content, good membrane integrity, low ion leakage, and hydrogen peroxide. Additionally, this treatment led to the improvement of photosynthetic pigments and antioxidant activity. The analysis of seedlings under cold stress showed that the Phe enhanced the stomatal conductance and phenol content. Furthermore, low concentrations of Cys resulted in the production of proline and flavonoids, which reduced the negative effects of environmental stress on seedlings and maintained cell membrane integrity. Overall, in this experiment, the use of Se and low concentrations of Cys had a positive effect on the amount of antioxidant compounds, which improved seedling growth under stress conditions.

Keywords: chlorophyll; cold stress; malondialdehyde; proline; *Physalis peruviana*

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1. Introduction

Cape gooseberry (*Physalis peruviana* L.) is a perennial plant belonging to the Solanaceae family, but it is grown commercially as an annual crop [1]. These fruits are highly beneficial to human health due to their nutritional and bioactive compounds, which help reduce the risk of diseases such as cancer, malaria, asthma, hepatitis, dermatitis, and rheumatism [2]. As a result, it has gained considerable attention for cultivation worldwide.

Environmental factors can have a negative impact on plant growth, development, and final performance [3]. Among these factors, low-temperature stress is one of the most challenging for agricultural crops, especially vegetables. Chilling temperatures (0–15 °C) during plant development, including seed germination, vegetative growth, and reproduction, can adversely affect summer vegetables [4]. Most of the damage caused to plants by cold stress is due to oxidative damage at the cellular level [5]. In stressful conditions, the production of reactive oxygen species (ROS) increases excessively, causing damage to macromolecules and negatively affecting plant growth [6]. The ability of plants to scavenge the toxic effects of ROS determines their tolerance to different stresses. Antioxidant enzymes such as catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD), as well as non-enzymatic antioxidants such as ascorbate, glutathione, and α-tocopherol, are associated with ROS scavenging in plants, and their synthesis is

known to be enhanced during exposure to oxidative stress [7]. Previous studies have shown that antioxidant machinery plays an essential role in cold tolerance. The induction of antioxidant systems, including enzymatic and non-enzymatic components, has been widely reported as an essential mechanism to control ROS accumulation under environmental stress conditions [8,9].

Low-temperature stress can cause various symptoms, including wilting, decreased plant growth and photosynthetic rate, chlorosis, necrosis, discoloration, abnormal ripening, increased susceptibility to diseases, decreased membrane permeability, increased ion leakage, and changes in respiration and ethylene production in plants [10,11]. Low-temperature stress decreases membrane fluidity, reduces water uptake and changes the balance between transpiration and water uptake, and leads to inadequate moisture in plant shoots [12]. Previous studies have shown that cucumber seedlings exposed to low light intensity and temperature stress below the desired level exhibit a decrease in ribulose 1,5-biphosphate carboxylase activity and chlorophyll content and an increase in malondialdehyde (MDA) content and lipid peroxidation compared to control plants [13].

Generally, it is believed that amino acids may induce cold stress tolerance in plants, as they can help regulate water status by increasing stomatal conductance and promoting root growth. L-phenylalanine (Phe), an essential amino acid, is commonly used as a nutritional enhancer and for amino acid injection and complex amino acid preparation [14]. The foliar application of amino acids such as L-phenylalanine during plant growth increased anthocyanins and phenolic compound contents in grape [15] and strawberry (*Fragaria* × *ananassa* Duch) [16] fruits. The irrigation of tomato (*Solanum lycopersicum* L.) seedlings with a nutrient solution supplemented with amino acids, i.e., alanine, serine, phenylalanine, and tyrosine, improved the leaf mineral status and chlorophyll concentration [17].

Cysteine (Cys) is an amino acid that contains sulfur, which is widely present in bacteria, yeast, plants, animals, and certain single cells. It is synthesized from the serine amino acid in the cytosol, plastid, and mitochondria, and it serves as a precursor to proteins, vitamin cofactors, and antioxidants such as glutathione and some defensive compounds in plants [18]. The postharvest application of cysteine in plum fruits improved antioxidant enzyme activity, reduced the hydrogen peroxide (H_2O_2) content, and maintained membrane integrity [19].

Selenium (Se) is a beneficial element for plants and can have positive effects on human health [20]. Se and its salts can protect plants from biotic (pathogens and herbivores) and abiotic (ultraviolet rays, heavy metals, arsenic) stresses by inhibiting lipid peroxidation and increasing the activity of glutathione peroxidase [21]. The foliar application of sodium selenate at low concentrations (3 and 6 mg L^{-1}) in sorghum seedlings under cold stress led to increased growth, chlorophyll, anthocyanin, and proline contents, increased enzymatic and non-enzymatic antioxidant capacity, and reduced lipid peroxidation by decreasing the MDA level [22]. The foliar spray of sodium selenate in strawberry seedlings alleviated the decline in the net photosynthetic rate and chlorophyll content and decreased the increase in MDA and H_2O_2 contents under low-temperature stress [23].

Despite several studies on the response of tropical and subtropical vegetables to cold stress, the effect of low-temperature stress on Cape gooseberry and the impact of foliar-applied amino acids on chilling-stressed Cape gooseberry remains unclear. Therefore, this study aimed to investigate the physiological response of Cape gooseberry seedlings subjected to chilling stress following the foliar application of two amino acids (L-phenylalanine and L-cysteine) and sodium selenate.

2. Materials and Methods

2.1. Plant Materials and Experimental Treatments

The present experiments were conducted in the Research greenhouse at the University of Zanjan, Zanjan, Iran (36°40′ N Long, 48°24′ E Lat, 1596.3 m altitude) during March 2021 and March 2022. The seeds of Cape gooseberry (*Physalis peruviana* L.) were provided by

Pakan Seed Company, Isfahan, Iran. The Cape gooseberry seeds were sown in seedling trays containing peat moss at one seed per cell. The seedlings were grown under optimal ambient conditions of 25 ± 2 °C during the day and 20 ± 2 °C at night, with a relative humidity of 60–65%, and an average photoperiod of 11.5 h and 7.5 h of sunshine. The seedlings were irrigated with Hoagland nutrient solution. The experiment was conducted in a completely randomized design (CRD) with three replicates, with 10 seedlings per replicate. For each treatment, 30 seedlings were used. Different concentrations of L-phenylalanine (0.75, 1.5, and 2.5 mM, Phe, Merck KGaA, Darmstadt, Germany), L-cysteine (0.25, 0.5, and 0.75 mM, Cys, Sigma-Aldrich, Munich, Germany), or sodium selenate (0.25, 0.5, and 1 mg L^{-1}, Se, Sigma-Aldrich, Munich, Germany) were sprayed on the seedlings at the 4–5th true leaf stage. Distilled water was used as the control treatment. To induce chilling stress, 300 seedlings with basically the same growth were transferred to a 4 °C climate chamber (16 h light/8 h dark with a relative humidity of 50–60%) for 48 h. A control group (30 seedlings), which was not treated with amino acid or Se and did not undergo chilling stress, was grown under optimal conditions of 25 ± 2 °C during the day and 20 ± 2 °C during the night, with a 60–65% RH. After applying the chilling stress, the seedlings were returned to optimal growth conditions for 24 h.

2.2. Chlorophyll and Carotenoid Contents

Three leaves from three plants per replicate in each treatment were randomly collected to evaluate the chlorophyll and carotenoid contents. Chlorophyll and carotenoids were extracted from 0.5 g of fresh leaves using 80% (*v*/*v*) acetone. The absorbance of the extract was measured at 663 and 645 nm for chlorophyll and 480 and 510 nm for carotenoids using a Safas Monaco (RS 232) spectrophotometer. The concentrations of total chlorophyll and carotenoids were expressed as mg g^{-1} fresh weight (FW) and calculated using the following formula reported by Arnon [24]:

$$\text{Chlorophyll contents (mg g}^{-1}\text{ FW)} = [20.2\ (A_{645}) + 8.02\ (A_{663})] \times V/(W \times 1000)$$
$$\text{Carotenoids (mg g}^{-1}\text{ FW)} = [7.6\ (A_{480}) - 1.49\ (A_{510})] \times V/(W \times 1000)$$

2.3. Stomatal Conductance

The leaf stomatal conductance was randomly measured in three leaves of five plants per replicate in each treatment, between 10:00 AM and 2:00 PM, using a poromoter device (MK model, Delta-T, Cambridge, UK) and expressed as μmol m^{-2} s^{-1}.

2.4. Relative Water Content

To assess the relative water content (RWC), three expanded young leaves from five plants per treatment in triplicate were randomly collected. The fresh weight (FW) of mature leaves was recorded, and then the leaves were immersed in distilled water for 24 h. After 24 h, the turgid weight (TW) of the leaves was obtained, followed by drying the leaves in an oven at 65 °C for 48 h until their constant dry weight (DW) was achieved. The leaf RWC was calculated using the following formula reported by Ritchie et al. [25]:

$$\text{RWC (\%)} = (\text{FW} - \text{DW})/(\text{TW} - \text{DW}) \times 100.$$

2.5. Proline Content

The proline content was measured by an acid ninhydrin method, according to Bates et al. [26]. To this end, three leaves were separated from five representative seedlings in each plot. Proline was extracted from 0.5 g fresh leaf samples in a cold mortar with liquid nitrogen with 5 mL of sulfosalicylic acid 3% and estimated using the ninhydrin reagent. The absorbance of the fraction with toluene that aspired from the liquid phase was read at the 520 nm wavelength. The proline concentration was determined using a calibration curve and expressed as mg g^{-1} FW.

2.6. Malondialdehyde and H_2O_2 Contents

The content of malondialdehyde (MDA) was measured using the thiobarbituric acid method, according to Zhao et al. [27], with modification. Leaf samples for MDA measurements were sampled from the five seedlings. Fresh leaf tissue (1 g) was extracted with 5 mL of 10% trichloroacetic acid (TCA) and centrifuged at 10,000 rpm for 15 min. Then, 2 mL of the supernatant was mixed with 2 mL of 10% TCA containing 0.6 g thiobarbituric acid (TBA). The mixture was placed in a water bath at 100 °C for 20 min and then quickly cooled in an ice bath and centrifuged at 6000 rpm for 10 min. The absorbance of the aqueous phase was measured at 532 nm and 600 nm using a spectrophotometer (Specord 250 Jena-History, Analytik Jena GmbH, Jena, Germany). The amount of MDA was calculated as μmol g^{-1} FW, where 1 unit was defined as 1 μmol MDA per gram of plant. Hydrogen peroxide (H_2O_2) was measured spectrophotometrically after reacting with potassium iodide (KI), as described by Alexieva et al. [28]. The fresh leaf tissue (1 g) was homogenized with 5 mL of 0.1% (*w*/*v*) TCA and centrifuged for 15 min at 10,000 rpm. Then, 0.5 mL of the supernatant was mixed with 0.5 mL of 10 mmol potassium phosphate buffer (pH 7.0) and 1 mL of 1 mol KI. The blank probe consisted of 0.1% TCA in the absence of leaf extract. The absorbance was measured at 390 nm. The H_2O_2 concentration of the supernatant was calculated using a standard curve prepared with known concentrations of H_2O_2.

2.7. Membrane Stability Index

To determine the membrane stability index (MSI) of leaves, four mature leaves of three plants per treatment at three replicates were randomly selected. Leaf samples were washed 3 times with distilled water to remove any surface contamination and then cut into 1 cm pieces and incubated (in a vial, at 25 °C) for 24 h. The electrical conductivity of the bathing solution (EC_1) was measured after incubation. Then, the same samples were autoclaved at 120 °C for 20 min, and a second reading of the electrical conductivity (EC_2) was made after cooling the solution to room temperature. The MSI was calculated using the following formula reported by Ezhilmathi et al. [29]: $MSI = (1 - EC_1/EC_2) \times 100$

2.8. Total Phenol and Flavonoid Contents

To determine the total phenol and flavonoid contents, three leaves from five plants per treatment in triplicate were randomly collected. Fresh leaf samples (2 g) were homogenized in 10 mL methanol/water (80:20, *v*/*v*). The total phenol content was assayed according to the Folin–Ciocalteu procedure [30]. Briefly, 0.1 mL of extract was mixed with 2 mL of 2% Na_2CO_3 and allowed to stand for 2 min at room temperature. For each sample, 0.1 mL of 50% (*v*/*v*) Folin–Ciocalteu reagent was added with mixing and allowed to stand for 30 min, and the absorbance of the mixture was recorded at 720 nm. The total phenol content was expressed as mg gallic acid equivalent (GAE) per 100 g FW. The total flavonoid content in the leaf extracts was determined using a colorimetric assay [31]. A total of 0.25 mL of extract was mixed with 75 μL $NaNO_2$ (5%). After 5 min, 0.15 mL of 10% aqueous $AlCl_3$ was added and vortexed. The mixture was allowed to stand for 6 min at room temperature. Then, 0.5 mL of 1 mol L^{-1} NaOH was added to this mixture. The final volume was adjusted to 2.5 mL with deionized water. The absorbance of the solution versus a blank at 510 nm was measured immediately. The results were expressed as mg of quercetin (QE) equivalents per 100 g FW.

2.9. Antioxidant Capacity

To determine the antioxidant capacity, one leaf per plant was sampled from five seedlings per treatment in triplicate. The free radical DPPH% (2,2-Diphenyl-1-picrylhydrazyl) scavenging activity was measured according to Dehghan and Khoshkam [32]. Fresh leaf tissue (2.0 g) was homogenated in methanol and then centrifuged. Leaf extracts (50 μL) were allowed to react with 1.95 mL of DPPH radical solution (0.1 mM in methanol) for 30 min. The decrease in absorbance was measured at 517 nm. The DPPH% reduction percentage was calculated

based on the following equation, where the Abs control is the absorbance of the DPPH% solution without the extract:

$$\% \text{ Inhibition of DPPH\%} = (\text{Abs control} - \text{Abs sample})/\text{Abs control} \times 100$$

2.10. Statistical Analysis

The experiments were performed using a completely randomized design (11 treatments including 3 Phe, 3 Cys, 3 Se, 1 chilling-treated seedling (CLT), and 1 control plant (not chilling-treated seedling), with 3 replications = 33 data, during 2 years). Data were statistically evaluated by a two-way ANOVA, according to a combined analysis of variance using SAS V9.3 (Statistical Analysis System; SAS Institute Inc., Cary, NC, USA) software, and means were compared by Duncan's multiple range tests at the 5% probability level. Values were expressed as mean ± SE (standard error), with n = 3.

3. Results and Discussion

3.1. Stomatal Conductance

The effects of Phe, Cys, and Se on stomatal conductance (g_s) under low-temperature conditions in two years are presented in Tables 1 and 2. The results showed that the g_s rate of seedlings subjected to 4 °C conditions (CLT) was significantly ($p \leq 0.01$) lower than that of the control. In both years, the foliar application of Phe, Cys, and Se improved the g_s rate under low-temperature conditions. The highest amount of g_s (0.827 μmol m^{-2} s^{-1}) was obtained in control seedlings in 2021 (Table 2). The interaction effect of the year by treatments had a significant effect ($p \leq 5\%$) on stomatal conductance (Table 1).

Table 1. The ANOVA for the effect of phenylalanine (Phe), cysteine (Cys), and sodium selenate (Se) foliar spray on the stomatal conductance, relative water content (RWC), total chlorophyll, carotenoid, phenol, flavonoid, proline, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents, antioxidant capacity, and membrane stability index (MSI) of Cape gooseberry seedlings under low-temperature conditions in two years (2021 and 2022).

Significance	Year	Treatment	Year × Treatment
Stomatal conductance	*	**	**
RWC	**	**	**
Total Chlorophyll	**	**	**
Carotenoid	**	**	**
Total phenols	**	**	**
Flavonoids	**	**	**
Antioxidant capacity	**	**	**
Proline	*	**	**
MDA	**	**	**
H_2O_2	ns	**	**
MSI	**	**	*

Note: ns, *, **, non-significant or significant at $p < 0.05$ or 0.01, respectively.

Temperature is one of the important factors that regulate stomatal movements and transpiration in plants. At low temperatures, stomata tend to close, which interferes with the regulation of stomatal conductance (g_s) in plants [33]. Xu et al. [34] reported that stomatal conductance in two *Camellia* species was reduced under chilling stress. Amino acids have numerous benefits, including increasing fertilizer uptake, facilitating water and nutrient uptake, and improving plant photosynthesis. Geshnizjani and Khosh-Khui [35] indicated that amino acid treatment led to a significant improvement in stomatal conductance, which shows the role of amino acids as inducers of acetyl CoA in the physiological processes of plants. The exogenous application of amino acids under cold stress, by increasing stomatal conductivity and especially by stimulating root growth, has been shown to lead to better regulation of the water status of plants, as reported in lettuce [3].

Table 2. The effect of phenylalanine (Phe), cysteine (Cys), and sodium selenate (Se) foliar spray on the stomatal conductance and relative water contents of Cape gooseberry seedlings under low-temperature conditions in two years (2021 and 2022). Data shown are mean values of n = 3, and the means followed by the same letter are not significantly different at 0.05 levels. Control: plants grown under optimal conditions. CLT: control plants exposed to low-temperature stress.

Treatment	Stomatal Conductance (μmol m^{-2} s^{-1})		Relative Water Content (%)	
	2021	2022	2021	2022
CLT	0.173 ± 0.0120 j	0.290 ± 0.0030 i	64.05 ± 5.53 d	76.60 ± 4.76 bc
Control	0.827 ± 0.0260 a	0.516 ± 0.06 b–e	84.56 ± 2.02 ab	87.21 ± 0.66 ab
Phe 0.75	0.607 ± 0.0145 b	0.453 ± 0.0376 d–g	83.51 ± 3.98 ab	85.45 ± 1.06 ab
Phe 1.5	0.533 ± 0.012 bcd	0.463 ± 0.0367 c–f	79.88 ± 3.12 abc	89.74 ± 2.56 a
Phe 2.5	0.453 ± 0.0203 d–g	0.573 ± 0.0285 bc	83.69 ± 3.48 ab	86.50 ± 0.29 ab
Se 0.25	0.277 ± 0.0219 i	0.366 ± 0.0176 f–i	89.57 ± 2.33 a	85.52 ± 0.79 ab
Se 0.5	0.303 ± 0.0203 hi	0.416 ± 0.0328 e–h	88.80 ± 2.87 a	81.59 ± 0.48 abc
Se 1	0.377 ± 0.0291 f–i	0.353 ± 0.0176 f–i	90.40 ± 1.89 a	90.73 ± 1.82 a
Cys 0.25	0.510 ± 0.0186 b–e	0.363 ± 0.0503 f–i	84.26 ± 2.77 ab	83.95 ± 3.48 ab
Cys 0.5	0.340 ± 0.0115 ghi	0.303 ± 0.0115 hi	70.80 ± 0.92 cd	88.62 ± 0.58 a
Cys 0.75	0.600 ± 0.0176 b	0.623 ± 0.02 b	76.27 ± 3.56 bc	89.98 ± 0.29 a

3.2. Relative Water Content

The data in Tables 1 and 2 display the relative water content (RWC) of Cape gooseberry seedlings applied with different concentrations of Phe, Cys, and Se over two consecutive years. The RWC of seedlings grown in 2021 was significantly ($p \leq 0.01$) lower than the control under low-temperature stress conditions. As the results show, the foliar application of Phe, Se, and Cys improved the RWC in seedlings exposed to cold stress.

Previous research has demonstrated that the application of Se at low concentrations is effective in improving plant growth and abiotic stress tolerance [36]. Selenium can regulate water status in plants, increase water absorption and tissue hydration during environmental stress, and help with the more effective absorption of water by the roots while reducing the loss of water in tissues by reducing transpiration [37]. In previous studies, it was announced that the application of Se led to an increase in the RWC in quinoa (*Chenopodium quinoa* Willd) plants [37].

3.3. Chlorophyll and Carotenoid Contents

Tables 1 and 3 display the total chlorophyll and carotenoid contents of seedlings treated with different concentrations of Phe, Cys, and Se in two years under low-temperature conditions. As the results show, the chlorophyll and carotenoid contents of seedlings grown in 2022 were significantly higher than that of seedlings grown in 2021. The low-temperature stress significantly ($p \leq 0.01$) decreased the total chlorophyll content compared to the control seedlings in 2021. The effect of Phe, Cys, and Se on the total chlorophyll and carotenoid contents depended on the concentration of treatments. The highest chlorophyll content (1.94 mg g^{-1} FW) was obtained in plants treated with 0.25 mg Se L^{-1}, and the highest content of carotenoids (0.66 and 0.65 mg g^{-1} FW) was observed in seedlings treated with 0.25 mg Se L^{-1} and 0.75 mM Cys in 2022, respectively (Table 3).

Our results are consistent with previous studies, such as Abbas [22], who showed that the application of sodium selenate at low concentrations increased the photosynthetic pigment content in sorghum-stressed plants. Lu et al. [38] reported that the chlorophyll a, b, and total chlorophyll contents of tomato seedlings significantly decreased under low-temperature conditions. Similarly, in this study, low-temperature stress had a significant negative effect on the chlorophyll content in Cape gooseberry seedlings, leading to lower values than those in plants kept under optimal conditions.

Table 3. The effect of phenylalanine (Phe), cysteine (Cys), and sodium selenate (Se) foliar spray on the total chlorophyll and carotenoid contents of Cape gooseberry seedlings under low-temperature conditions in two years (2021 and 2022). Data shown are mean values of n = 3, and the means followed by the same letter are not significantly different at 0.05 levels. Control: plants grown under optimal conditions. CLT: control plants exposed to low-temperature stress.

Treatment	Total Chlorophyll (mg g^{-1} FW)		Carotenoid (mg g^{-1} FW)	
	2021	2022	2021	2022
CLT	0.89 ± 0.027 hi	1.49 ± 0.037 c–f	0.355 ± 0.022 def	0.508 ± 0.036 bc
Control	1.21 ± 0.034 k	1.42 ± 0.013 efg	0.332 ± 0.040 ef	0.544 ± 0.023 b
Phe 0.75	1.15 ± 0.016 ij	1.58 ± 0.034 bcd	0.351 ± 0.013 def	0.436 ± 0.021 c
Phe 1.5	1.15 ± 0.073 ij	1.39 ± 0.021 fg	0.324 ± 0.008 ef	0.536 ± 0.002 b
Phe 2.5	1.08 ± 0.037 ij	1.62 ± 0.042 bc	0.379 ± 0.010 de	0.492 ± 0.022 bc
Se 0.25	1.04 ± 0.01 j	1.94 ± 0.052 a	0.366 ± 0.016 def	0.663 ± 0.035 a
Se 0.5	1.17 ± 0.018 ij	1.32 ± 0.085 gh	0.273 ± 0.004 f	0.472 ± 0.020 bc
Se 1	1.16 ± 0.046 ij	1.66 ± 0.039 b	0.321 ± 0.031 ef	0.349 ± 0.022 d
Cys 0.25	1.03 ± 0.004 j	1.47 ± 0.018 def	0.317 ± 0.030 ef	0.541 ± 0.020 b
Cys 0.5	1.09 ± 0.049 ij	1.56 ± 0.027 b–e	0.362 ± 0.022 def	0.545 ± 0.013 b
Cys 0.75	1.16 ± 0.023 ij	1.57 ± 0.010 bcd	0.359 ± 0.009 def	0.653 ± 0.029 a

Chlorophyll decreases when plants are subjected to cold stress due to the increased production of reactive oxygen species (ROS) and oxidative stress that damage chloroplast membranes and thylakoid structures [39]. However, Se, due to its antioxidative role, photovoltaic function, and effects on photopigments, can counteract the harmful effects of oxidative stress [36]. Different concentrations of Se foliar sprays can mitigate chlorophyll degradation in chili pepper (*Capsicum annuum* L.) leaves due to cold stress [40]. Moreover, amino acid treatments such as proline and cysteine have been reported to modulate the harmful effect of cold stress on photosynthetic pigments and improve enzyme activity in wheat plants [41]. Takahashi et al. [42] reported that the promotive effect of cysteine in increasing photosynthetic pigments might be due to the fact that the chloroplast is the main source of sulfide via sulfate reduction in the sulfur assimilation pathway. Amino acid application significantly increased the total chlorophyll and carotenoid contents of leafy cabbage (*Brassica oleracea* var. capitata) compared to control plants [43].

3.4. Proline Content

The statistical analysis revealed that the year and treatments had significant effects on the proline content (Table 1). Based on the results, low-temperature stress had no significant effect on the proline content compared to control seedlings in both years (Figure 1). The application of Cys, 0.5 mg Se L^{-1}, and 1.5 mM Phe significantly promoted the accumulation of proline in 2021 (Figure 1). Also, in 2022, the foliar application of Phe, Se, and Cys at 0.25 and 0.5 mM significantly ($p \leq 0.01$) increased the proline content compared to CLT and control seedlings. The maximum proline content (0.516 and 0.441 mg g^{-1} FW) was observed in seedlings treated with 0.25 mM Cys and 0.5 mg Se L^{-1}, respectively, in 2021.

Proline, as a proteinogenic amino acid in plants, naturally increases during abiotic stresses by increasing proline synthesis or decreasing proline degradation [44]. Yang et al. [45] observed an increase in the proline content in tomato seedlings by decreasing temperature. Stressful conditions lead to the overproduction of proline, which helps maintain the osmotic balance or cell turgor pressure and stabilize membranes, thereby preventing ion leakage, keeping the ROS concentration within regular limits, and preventing an oxidative burst in plants under stress [46]. Yang et al. [47] suggested that various types of tomato plants may have different mechanisms for tolerating low night-time temperatures, perhaps because of different patterns of proline accumulation between plants. Shekari and Jawanmandi [48] stated that the application of cysteine has a vital and important role in the cytosol and mitochondria of plant cells, and its existence is necessary for the growth of hair roots. The

highest proline content was observed in gerbera (*Gerbera jamesonii* L.) flowers sprayed with 0.5 mg L^{-1} of an amino acid mixture [35]. Also, more recently, Singh et al. [49] showed that the accumulation of proline has been correlated to plant stress tolerance as an ROS absorber and plays an important role against oxidative damage.

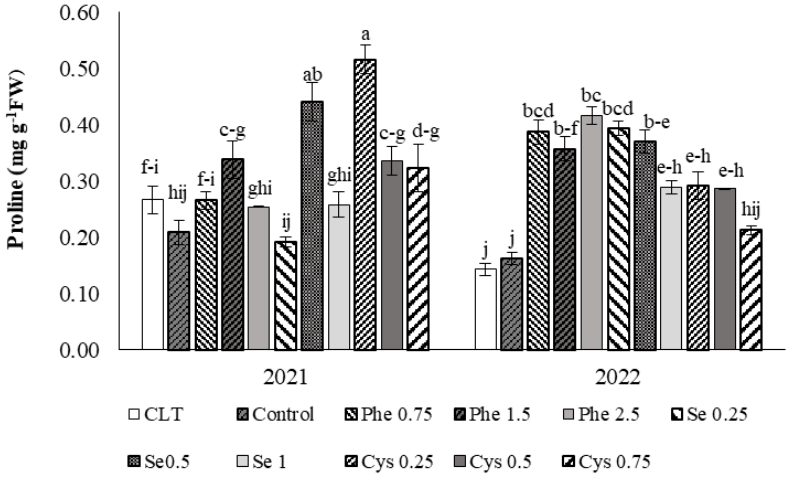

Figure 1. The effect of phenylalanine (Phe), cysteine (Cys), and sodium selenate (Se) foliar spray on the proline content of Cape gooseberry seedlings under low-temperature conditions in two years (2021 and 2022). Data shown are mean values of n = 3, and the means followed by the same letter are not significantly different at 0.05 levels. Control: plants grown under optimal conditions. CLT: control plants exposed to low-temperature stress.

3.5. Malondialdehyde and Hydrogen Peroxide Contents

As shown in Table 1 and Figure 2, the MDA and H_2O_2 contents in CLT seedlings were significantly higher than the control and most treated seedlings. Based on the results, the MDA and H_2O_2 contents of CLT and control seedlings in the second year were significantly higher than in the first year. In the first year, the exogenous application of Phe, Cys, and Se significantly decreased the MDA accumulation compared to CLT seedlings, although no significant difference was observed between CLT seedlings and high levels of Phe (2.5 mM) and Se (1 mg L^{-1}). However, only the application of 0.75 and 2.5 mM Phe and 1 mg Se L^{-1} caused a significant decrease in the MDA content compared to CLT seedlings in 2022. The minimum value of MDA (10.74 and 11.07 µmol g^{-1} FW) content was observed in seedlings treated with 0.25 mM Cys and control seedlings in 2021 (Figure 2A). The effect of Phe, Cys, and Se on H_2O_2 content depended on the level of treatments (Figure 2B). The lowest H_2O_2 content (2.85 µmol g^{-1} FW) was obtained from seedlings treated with 0.5 mg Se L^{-1} in 2022.

Malondialdehyde is known and investigated as a cytotoxic chemical produced by lipid peroxidation and as an oxidative marker in many studies related to plants under stress [50]. Under cold stress conditions, excessive free radicals in the plant cause damage to the membrane. The reaction occurs on the membrane structure, which is membrane lipid peroxidation, and the final product is MDA [45]. Similar results were reported by Aghdam et al. [51] in tomato fruits, where MDA and H_2O_2 accumulation increased during storage at 4 °C, and the exogenous application of Phe at 5 mM displayed significantly lower MDA and H_2O_2 accumulation compared to the control. According to Nasibi et al. [6], the application of cysteine in *Polianthes tuberosa* L. reduced the MDA content and electrolyte leakage. The beneficial role of cysteine in decreasing H_2O_2 and MDA contents might be attributed to its products, such as glutathione, which has antioxidant activity and directly scavenges H_2O_2 and maintains the membrane integrity [52]. Moreover, plants treated with Se led to a decrease in H_2O_2 and MDA accumulation in pepper seedlings under low-temperature conditions [40]. Previous studies demonstrated that the Se supply under cold stress could increase the antioxidant capacity of wheat seedlings, and optimal Se accumulation reduced the generation of free radicals and membrane lipid peroxidation [53].

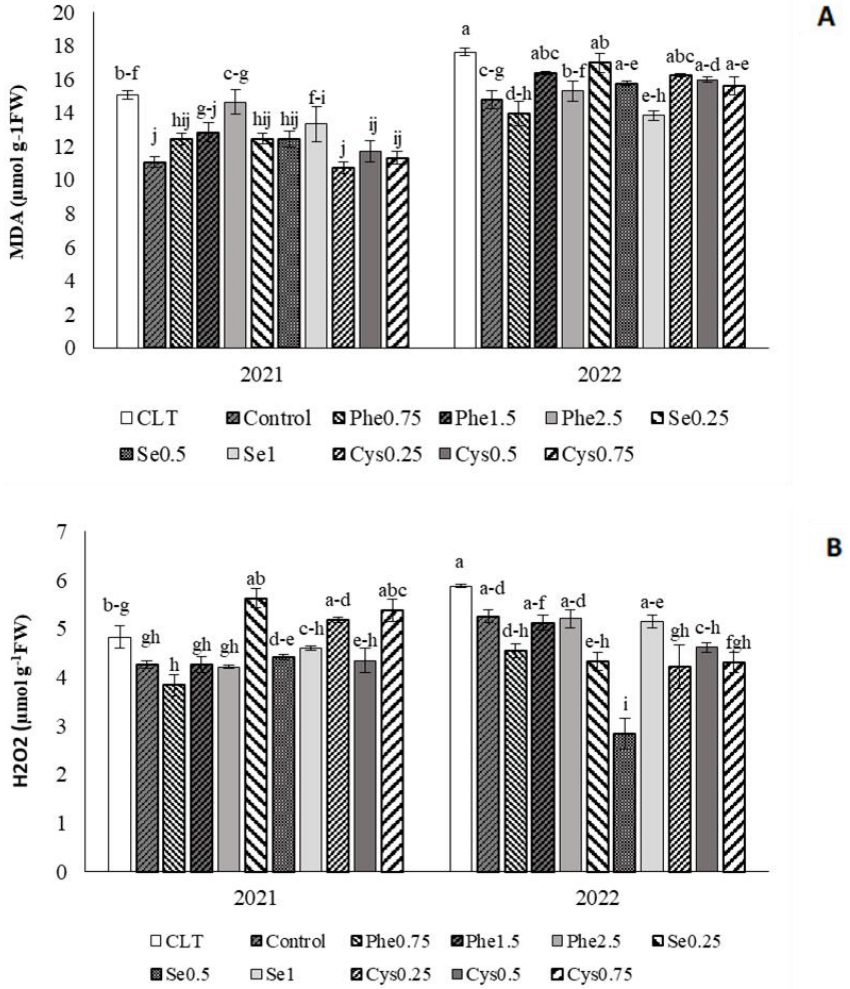

Figure 2. The effect of phenylalanine (Phe), cysteine (Cys), and sodium selenate (Se) foliar spray on the malondialdehyde (MDA) (**A**) and hydrogen peroxide (H_2O_2) (**B**) contents of Cape gooseberry seedlings under low-temperature conditions in two years (2021 and 2022). Data shown are mean values of n = 3, and the means followed by the same letter are not significantly different at 0.05 levels. Control: plants grown under optimal conditions. CLT: control plants exposed to low-temperature stress.

3.6. Membrane Stability Index

The data presented in Table 1 and Figure 3 show the membrane stability index (MSI) of seedlings treated with different concentrations of Phe, Cys, and Se over two years under low-temperature conditions. The statistical analysis revealed that the year and treatments had a significant effect on the MSI (Table 1). The results indicate that cold stress caused a significant decrease in the seedling MSI in 2021. The MSI of seedlings in 2021 was higher than in 2022, and the maximum value of the MSI was observed in seedlings treated with all Phe, Se, and Cys treatments and the control seedlings in the first year (Figure 3).

Under stress conditions, the accumulation of ROS can cause oxidative damage to plant cells, leading to significant damage to cellular components such as membrane lipids [54]. Our experiment shows that Se significantly reduced MDA accumulation and the negative effects of ROS on Cape gooseberry seedling membranes, which led to a significant improvement in plant growth under low-temperature conditions. Filek et al. [55] found that the use of Se for stress can restore the lipid and fatty acid composition of plastid envelope membranes. Additionally, the activity of enzymes that are important for membrane function can be reactivated and restored with its use. Based on these results, preventing MDA accumulation and ion leakage may be related to the improvement of cell membrane integrity in response to Se, which is probably caused by the activity of antioxidant systems [56].

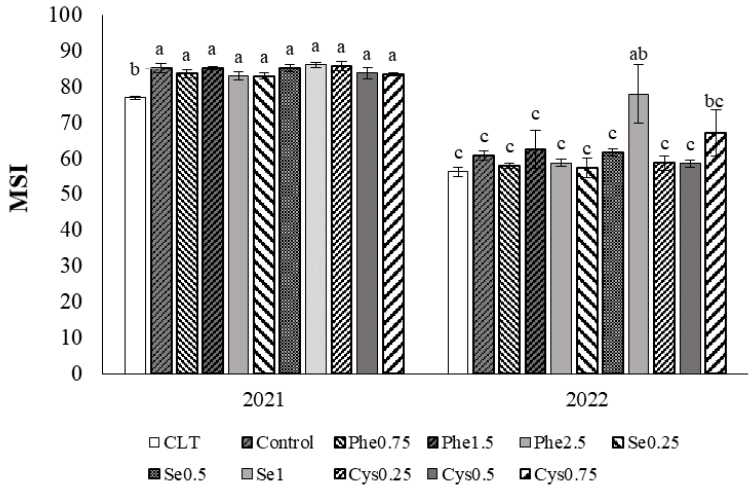

Figure 3. The effect of phenylalanine (Phe), cysteine (Cys), and sodium selenate (Se) foliar spray on membrane stability index (MSI) of Cape gooseberry seedlings under low-temperature conditions in two years (2021 and 2022). Data shown are mean values of n = 3, and the means followed by the same letter are not significantly different at 0.05 levels. Control: plants grown under optimal conditions. CLT: control plants exposed to low-temperature stress.

Aghdam et al. [51] reported that the use of Phe in tomato fruits induced tolerance to chilling damage, thus maintaining membrane integrity, which is associated with a lower percentage of electrolyte leakage and MDA accumulation. Nasibi et al. [6] also found that a cysteine solution reduced the MDA content and ion leakage.

3.7. Total Phenol and Flavonoid Contents

Based on the results, the interaction effect of the year by treatments had a significant effect ($p \leq 0.01$) on total phenol and flavonoid contents (Table 1). Different patterns for total phenol and flavonoid contents were observed between two years. The positive effects of Phe, Cys, and Se on the total phenol and flavonoid contents were dependent on the treatment levels, where the maximum content of total phenols (44.6 mg GAE 100 g^{-1} FW) was obtained in seedlings treated with 2.5 mM Phe in 2022 (Table 4). Different trends for the flavonoid content were observed among the treatments in two years, so the seedlings applied with 0.25 and 0.75 mM Cys and 0.5 mg Se L^{-1} showed the highest flavonoid content (14.08, 13.27, and 12.17 mg QE 100 g^{-1} FW) in 2021 (Table 4).

During low-temperature stress, the photosynthesis rate is inhibited, and the production of ROS is stimulated, which, in turn, damages the cells [57]. Plants accumulate antioxidant metabolites, such as phenolic compounds, during low-temperature stress and develop stress resistance ability [58]. As our results indicated, the total phenol and flavonoid contents increased in Cape gooseberry seedlings during low-temperature stress. Similar to our findings, cold stress induced the accumulation of phenolic compounds in kimchi cabbage (*Brassica rapa* L. ssp. Pekinensis) plants [9]. Rana and Bhushan [59] also suggested that low-temperature stress induces the biosynthesis of phenolic compounds in plants and provides tolerance against cold stress. Amino acids are important components of plant antioxidant systems. In the present study, pretreatment with Cys, Phe, and Se increased phenolic compounds during low-temperature conditions. Such findings were reported by the authors of [60], who stated that Phe foliar application increased total phenol and flavonoid contents in moringa (*Moringa oleifera*) leaves. Ardebili et al. [61] stated that the foliar spray of amino acids induced phenylalanine ammonia-lyase activity and thus significantly improved the phenol content in *Aloe vera* L. plants. Rios et al. [62] reported that the application of Se in lettuce plants increased the total flavonoid content. Similarly, Bachiega et al. [63] found that broccoli's phenolic compounds significantly increased after being biofortified with Se, and the antioxidant activity also improved.

Table 4. The effect of phenylalanine (Phe), cysteine (Cys), and sodium selenate (Se) foliar spray on the total phenol and flavonoid contents and antioxidant capacity of Cape gooseberry seedlings under low-temperature conditions in two years (2021 and 2022). Data shown are mean values of n = 3, and the means followed by the same letter are not significantly different at 0.05 levels. Control: plants grown under optimal conditions. CLT: control plants exposed to low-temperature stress.

Treatments	Total Phenols (mg GAE g^{-1} FW)		Flavonoids (mg QE g^{-1} FW)		Antioxidant Activity (%)	
	2021	2022	2021	2022	2021	2022
CLT	24.19 ± 1.86 hij	31.07 ± 1.29 def	6.24 ± 0.23 ef	6.17 ± 0.35 ef	83.03 ± 1.93 ab	49.62 ± 0.85 hi
Control	20.49 ± 2.502 j	25.65 ± 0.67 ghi	4.69 ± 0.66 f	5.27 ± 0.12 ef	70.07 ± 0.24 cd	45.98 ± 0.42 i
Phe 0.75	23.46 ± 1.01 hij	33.12 ± 0.88 cde	5.11 ± 0.43 ef	8.93 ± 0.36 cd	75.11 ± 0.33 bc	76.30 ± 4.44 bc
Phe 1.5	22.26 ± 1.38 ij	28.25 ± 0.85 e–h	4.89 ± 0.27 ef	5.94 ± 0.11 ef	80.40 ± 2.24 ab	59.26 ± 2.79 efg
Phe 2.5	26.25 ± 0.97 f–i	44.68 ± 0.04 a	7.04 ± 0.66 de	9.23 ± 0.45 c	79.44 ± 2.14 ab	49.55 ± 0.99 hi
Se 0.25	38.22 ± 0.43 bc	26.25 ± 0.64 f–i	11.59 ± 0.62 b	4.98 ± 0.53 ef	87.47 ± 1.45 a	52.59 ± 0.14 f–i
Se 0.5	38.18 ± 0.305 bc	25.67 ± 0.56 ghi	12.17 ± 0.61 ab	5.27 ± 0.39 ef	86.33 ± 1.88 a	59.00 ± 0.85 efg
Se 1	32.50 ± 1.42 de	34.92 ± 0.60 bcd	11.92 ± 0.87 b	8.57 ± 0.38 cd	81.96 ± 2.43 ab	58.75 ± 4.20 e–h
Cys 0.25	35.42 ± 1.74 bcd	39.07 ± 0.82 b	14.07 ± 0.34 a	8.48 ± 0.13 cd	85.90 ± 1.13 a	60.96 ± 0.61 ef
Cys 0.5	35.40 ± 1.54 bcd	29.37 ± 0.60 efg	12.05 ± 0.75 b	5.93 ± 0.19 ef	82.92 ± 3.81 ab	50.19 ± 1.59 ghi
Cys 0.75	36.25 ± 1.27 bcd	34.77 ± 1.76 bcd	13.27 ± 0.66 ab	6.05 ± 0.59 ef	80.97 ± 2.37 ab	65.08 ± 2.49 de

3.8. Antioxidant Capacity

The antioxidant capacity showed a significant difference between the two years, which was higher in 2021 than in 2022 (Tables 1 and 4). The results of this study indicate that low-temperature stress and Phe, Se, and Cys treatments increased the antioxidant capacity of Cape gooseberry seedlings compared to control seedlings in 2021 (Table 4). The highest values (87.4%, 86.3%, and 85.9%) were obtained in seedlings treated with 0.25 and 0.5 mg Se L^{-1} and 0.25 mM Cys in 2021, respectively (Table 4).

To alleviate oxidative stress injury, plants have evolved mechanisms to scavenge ROS through the antioxidation of enzymatic and non-enzymatic systems [23]. Barzegar et al. [8] reported a strong relationship between antioxidant activity (DPPH scavenging activity) and the carotenoid, total phenol, and flavonoid contents in sweet pepper (*Capsicum annuum* L.). Eom et al. [9] indicated that cold-induced polyphenolic compounds improve free-radical scavenging activity and antioxidant capacity, suggesting that the phenylpropanoid pathway induced by cold stress contributes to resistance to cold-induced reactive oxygen species in kimchi cabbage. As the results indicate, the beneficial role of Se and amino acids in increasing the antioxidant capacity might be attributed to enhancing phenolic compounds and carotenoid content, which have antioxidant activity. These results agree with Wen et al. [64], who showed that the use of cysteine increased free-radical scavenging activity (DPPH) in plants. In addition, it has been reported that the application of Phe significantly increased the antioxidant activity in moringa leaves [60]. Exogenous Se application on chili (*Capsicum annuum* L.) leaves significantly increased enzymatic and non-enzymatic antioxidant contents against ROS generation due to cold stress [40]. Moreover, Huang et al. [23] reported that the exogenous application of Se on strawberry seedlings reduced cold stress due to increased antioxidant levels.

4. Conclusions

In summary, the results from this study showed that low-temperature stress induces significant physiological and biochemical changes in Cape gooseberry seedlings. Low temperatures triggered a higher accumulation of MDA and H_2O_2 and a lower MSI and stomatal conductance than non-chilling conditions. Also, low-temperature stress leads to an increase in the proline content and antioxidant capacity of seedlings. The foliar

application of Cys, Phe, and Se enhanced the accumulation of proline, the stomatal conductance, and the RWC. The higher proline content and antioxidant activity contributed to reductions in the MDA and H_2O_2 accumulation in Cape gooseberry seedlings under chilling stress conditions, which maintained the stability of membranes and improved the seedling's low-temperature tolerance. Based on these results, we can conclude that the effects of the Phe, Cys, and Se on Cape gooseberry seedling were different. The exogenous use of Cys and Se at low concentrations is beneficial for the growth of Cape gooseberry seedlings, especially under stress conditions, but Phe could potentially be beneficial at higher concentrations.

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