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Original Research Paper

Foliar application of sucrose and Bordeaux mixture enhances freezing tolerance in radish (*Raphanus sativus* L.)

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Abstract: Although radish is considered a cool-season vegetable, freezing temperatures can cause serious problems for its growth. To enhance radish tolerance to freezing stress, an experiment was conducted in a completely randomized factorial design. This design incorporated two factors: sucrose concentration (zero and 0.5%) and anti-stress compounds (control, 1 mM salicylic acid, 0.1 mM sodium nitroprusside (SNP), and 0.5% Bordeaux mixture), with four replications for each treatment. The findings indicated that plants sprayed with a combination of sucrose and Bordeaux mixture exhibited the highest storage hypocotyl flavonoid content (0.125 µg Quercetin/mg extract). This value was not significantly different from that observed in plants treated with SNP alone or with sucrose alone (without any anti-stress compounds). lants treated with Bordeaux mixture alone (without sucrose) showed a higher membrane stability index (94.33%), which was significantly different only from the control and salicylic acid treatments. Plants treated with Bordeaux mixture + sucrose- showed the lowest storage hypocotyl lipid peroxidation and the highest chlorophyll b content (0.928 mg/g FW) and total chlorophyll (2.976 mg/g FW) in the leaves. The highest hypocotyl lipid peroxidation was observed in control plants and in plants treated with salicylic acid alone. Control plants (no sucrose or anti-stress compounds) and plants treated with salicylic acid displayed the highest storage hypocotyl proline content. There was a significant negative correlation between leaf proline and storage hypocotyl lipid peroxidation (r=-0.85) and storage hypocotyl flavonoid and lipid peroxidation (r=-0.74). In addition, we found a significant negative correlation between leaf membrane stability percentage and storage hypocotyl lipid peroxidation (r=-0.79). In conclusion, this study demonstrates that most of the compounds tested could enhance the storage hypocotyl's tolerance to freezing stress. However, the combination of sucrose and Bordeaux mixture proved to be the most effective and warrants further investigation in future research.

Key words: Carbohydrate; low temperature; membrane stability; nitric oxide; salicylic acid.

1. Introduction

Radish (*Raphanus sativus* L.) is an annual cool-season plant, and the best storage hypocotyl quality is obtained at a temperature of 10 to 18 °C (Abby, 2013). Low temperature is one of the important problems that can limit the growth and development of all types of crops in the cool season, especially in dry and temperate regions (Nezami et al., 2016). At low temperatures, the activity of proteins involved in maintaining cell structure and function decreases because the intercellular water turns into ice, and this reduces the movement of water. Furthermore, low temperatures can cause lipid oxidation of cell membrane since it can damage membrane structure and protein destruction induced by reactive oxygen species (ROS) (Grant et al., 2014). Freezing stress can increase membrane electrolyte leakage and decrease cell swelling in plant tissues, Thus, plant membranes are the first target of freezing stress, where membrane constituents play different roles in abiotic and biotic stress sensing (Adigun et al., 2021). Proline has been reported to increase under various environmental stresses, including low-temperature stress (Cook et al., 2004).

Ice-active bacteria are a group of bacteria that can catalyse ice formation at sub-zero temperatures and cause damage to plants under freezing stress (Waturangi and Tjhen, 2009). The formation of ice crystals inside the protoplasm causes breakage and damage to the cell and may lead to the death of the plant. Several compounds are reported to help maintaining membrane integrity in plants under stress conditions. Bordeaux mixture is a copper-based compound that is used as the conventional treatment against fungi and bacteria by killing ice-forming bacteria, to increase the resistance of plants and reduce the physiological disorders caused by freezing stress (Gutierrez-Barranquerovet al., 2012). Salicylic acid (SA), as a pivotal signalling molecule, plays a crucial role in plant tolerance responses to both biotic and abiotic stresses (Song et al., 2023). SA moderates the negative impact of various stresses, including freezing, by affecting peroxidase and catalase enzymes as well as osmotic regulators such as proline, glycine, and betaine (Dat et al., 2000), which reduces reactive oxygen species (ROS) and increases the resistance of plants. Additionally, salicylic acid can increase plant hormones such as auxins and cytokinins, thereby reducing ion leakage from plant cells (Shakirova et al., 2003).

Sodium nitroprusside (SNP) a nitric oxide (NO) donor, has a variety of impacts on plant physiology. NO is involved in cell metabolism and morphogenesis and acts as a signalling molecule in response to various biotic and abiotic stresses (Karthik et al., 2019). It has been reported that the use of nitric acid in cucumber seedlings under low-temperature stress enhances plant adaptation to cold stress by increasing the activity of protective enzymes (Liu et al., 2011). SNP can enhance seed germination rate, relative water content, proline levels, and chlorophyll concentration in corn plants under low-temperature stress. Furthermore, SNP can decrease cell membrane electrolyte leakage (Chen et al., 2012).

Soluble carbohydrates in plants are important for creating resistance to freezing stress. The use of exogenous carbohydrates, combined with other anti-stress compounds, can therefore enhance freezing resistance. In this regard, Jacobsen et al. (2007) reported that the variation in frost tolerance is due to the difference in the amount of soluble carbohydrates. The high content of soluble carbohydrates acts as an osmosis regulator and a cold protector and helps stabilize the membranes (Sevillano et al., 2009).

This experiment was conducted to investigate the effects of foliar spraying of sucrose and various anti-stress compounds (including salicylic acid, Bordeaux mixture and SNP) on freezing stress tolerance in radish, by examining physiological aspects such as membrane stability, proline levels, and photosynthetic pigments.

2. Materials and method

2.1. Plant materials and growth conditions

A greenhouse experiment was conducted in a completely randomized factorial design, with two factors and four replications in the research greenhouse of the Sari University of Agricultural Sciences and Natural Resources, Sari, Iran. The first factor comprised two levels of sucrose (0 and 0.5%), and the second factor included four anti-stress compounds (control (Water), 1 mM salicylic acid, 0.1 mM sodium nitroprusside (SNP), and 0.5% Bordeaux mixture). Sucrose concentration was selected following the indication of previous studies (Akbarpour et al., 2024; Mashayekhi and Atashi, 2012). SNP concentration was adopted from Saeedi et al (2022) and Zangani et al (2023). The salicylic acid concentration was adopted from the literature (Zahid et al., 2023; Shahbazi et al., 2024). Bordeaux mixture concentration obtained from Nikkho et al., (2024).

The seeds were sown in plastic pots with dimensions of 85 × 75 mm, filled with a mixture of coir fibre and perlite in a 70:30 ratio by volume. The foliar spraying was applied two weeks after sowing the seeds, exactly 48 hours before exposing the plants to freezing stress. The treatments (sucrose and the other anti-stress substances) were applied simultaneously by mixing and then spraying themon the plants.

Plants were placed in an incubator with a temperature of -4 °C for 12 hours overnight to induce freezing stress. The temperature was gradually lowered by 2 °C every hour. Then the temperature was

gradually raised to the ambient temperature (25 °C) to allow recovery and avoid sudden temperature changes (Lei et al., 2019). Cold stress was applied for only one night. After four hours at room temperature, biochemical measurements were taken on radish leaf and storage hypocotyl tissues.

2.2. Measurement of membrane stability index and lipid peroxidation

The method outlined by Sairam and Srivastava (2002) was used to measure the membrane stability index in both leaf and storage hypocotyl with three replications in each treatment. Briefly, 0.1 g of fresh leaf tissue was immersed in 10 mL of distilled water. The tube placed in a hot water bath at a temperature of 40 °C for 30 minutes. Following this, an EC meter model (Itrans instruments HC3010) was used to determine the electrical conductivity of the solution (EC_1), then samples were placed in a hot water bath at a temperature of 100 ºC for 15 minutes then electrical conductivity (EC_2) was measured. Finally, membrane stability index (MSI) was determined using the following equation (Eq.1)

$$MSI = 1 - (EC_1/EC_2) \times 100 \qquad (1)$$

Lipid peroxidation was determined by the malondialdehyde (MDA) method described by Heath and Packer, (1986). A 0.5 g of leaf and storage hypocotyl sample (three samples per plant and three replicates per treatment) was blended in 1 mL of 1% trichloroacetic acid. The mixture was subjected to centrifugation at 12,000 rpm for 15 min. Then, 500 µL of 20% trichloroacetic acid and 0.5% 2-thiobarbituric acid were added to 500 µL of the supernatant. The mixture was then heated to 95 °C for 30 minutes. After cooling, the tubes were centrifuged for 5 min at 10,000 rpm. A spectrophotometer was used to measure the absorbance at 532 nm, which was then adjusted by subtracting the absorbance at 600 nm. The malondialdehyde (MDA) content was calculated based on the MDA extinction coefficient (155 $mM^{-1}cm^{-1}$) and expressed as MDA equivalent (nmol g^{-1} fresh weight).

2.3. Determination of proline

The method described by Bates et al. (1973) was used to measure proline in leaves and storage hypocotyls with three replicates in each treatment First, 0.5 grams of each sample (leaf and storage hypocotyls) was ground with 10 mL of 3% sulfosalicylic acid in a mortar and centrifuged for 10 minutes at a speed of 2000 rpm. Then 2 mL of filtered extract were mixed with 2 mL of Ninhydrin reagent and 2 mL of acetic acid. The samples were placed in a bain-marie with a temperature of 100 ºC for one hour, and after cooling, 4 mL of toluene was added to them and stirred vigorously for 20 seconds. The optical absorbance of the supernatant solution was read with a spectrophotometer (Hanon instrument i3) at 520 nm wavelength. The spectrophotometer was calibrated with toluene and the standard curve was obtained with different concentrations of pure proline.

2.4. Measurement of chlorophyll and carotenoid

To determine leaf chlorophyll, extracts were prepared using 100% methanol as described by Carter and Knapp (2001). Six discs of leaf samples (first or second developed leaf) with three replicates in each treatment were put in a tube with 8 mL 100% methanol and left in darkness at 30 °C for 24 hours. The absorbance at wavelengths of 665.2, 652.4, and 470 nm ($A_{665.2}$, $A_{652.4}$, and A_{470}, respectively) was measured with a spectrophotometer (Hanon instrument i3) and the following equations (Lichtenthaler and Buschmann, 2001) were used to calculate the pigment concentrations:

$$\text{Chlorophyll a} : 16.72\ A_{665.2} - 9.16\ A_{652.4} \qquad \text{(Eq. 1)}$$

$$\text{Chlorophyll b} : 34.09\ A_{652.4} - 15.28\ A_{665.2} \qquad \text{(Eq. 2)}$$

$$\text{Carotenoid} : 1000\ A_{470} - (1.63\ \text{Chl. a}) - (104.96\ \text{Chl. b}) \ / \ 221 \qquad \text{(Eq. 3)}$$

2.5. Determination of antioxidant capacity, total phenol and flavonoids

One g of sample was mixed with10 mL of 80% (v/v) methanol and incubated at room temperature (25 °C) for 24 h, and the supernatant was collected after centrifugation at 2000 rpm for 10 min. This extract was used for measuring total phenols, total flavonoids an antioxidant capacity. Plant total flavonoids were estimated in leaf and storage hypocotyl samples using the aluminium chloride colorimetric method provided by Chang et al. (2002). Briefly, 0.5 mL of extract was mixed with 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 mol/L potassium acetate, and 2.8 mL of distilled water. Then the tubes were left at 25 °C for 30 minutes to incubate and the absorbance at 415 nm was measured using a spectrophotometer (Hanon instrument i3). Total flavonoids in samples were expressed as mg quercetin in the extract.

Total phenol content was assessed using the colorimetric Folin-Ciocalteu method. Briefly, 20 µL of the leaf and storage hypocotyl extract was added to a tube, and 1.6 mL of ultrapure water and 100 µL of 2-fold diluted Folin-Ciocalteu reagent were added. These were incubated for 5 min, at 37 °C, then 300 µL of saturated Na_2CO_3 (1 Mol) was added, and the mix was put for 30 minutes in a water bath set at 40 C. Then the absorbance at 765 nm was measured against a reagent blank using a spectrophotometer (Hanon instrument i3) and gallic acid was used as the reference standard to measure total phenol in samples (Laurie and Waterhouse, 2006). To measure antioxidant capacity, a DPPH reagent was used. Briefly1 mL of 0.1 mM reagent was added to 1 mL of the extract and then kept in a dark place for 15 minutes. Then, the absorption at 517 nm was read by a spectrophotometer (Hanon instrument i3) and the antioxidant activity was expressed as the percentage of inhibition of DPPH free radicals according to the following equation: (1) (Ebrahimzadeh et al., 2009).

$$\text{Equation (1): Inhibition (\%)} = [(Ac-As)/Ac] \times 100 \qquad \text{(Eq. 4)}$$

Where Ac is the control absorbance and As is the sample absorbance

2.6. Total soluble carbohydrates

To measure total soluble carbohydrates in leaves we used the method described by Sadasivam and Manickam (1992) and Franscistt et al. (1971). Briefly, 0.5 g of dried plant tissue was poured into 10 mL polyethylene tubes and 5 mL of 80% ethanol was added to the samples. The samples were placed in a water bath at 80 °C for 20 min and then centrifuged for 3 min at 2000 rpm. Then the supernatant was transferred to 15 mL tubes and extraction was repeated three times with ethanol 80%. Supernatants were collected into 20 mL volumetric flask. then 200 µL of the concentrated extract was pipetted to separate test tubes and then tubes were placed in the water bath at 80 °C to evaporate the ethanol in samples. Three mL of anthrone reagent was added to extracts and, after being placed in a water bath at 80 °C for 20 min, the absorbance at 620 nm of the extracts was read with a spectrophotometer (Hanon instrument i3). Total sugar content of the samples was calculated by comparison with the standard chart.

2.7. Statistical analyses

Data was subjected to analysis of variance (two-way ANOVA) using SAS software (ver. 9.1, SAS Institute, Cary, NC, USA). Means were separated using the Tukey test at $p<0.05$.

3. Results

3.1. Phytochemical compounds and membrane stability index

The analysis of variance showed that the interaction between sucrose foliar spraying and anti-stress compounds significantly affected storage hypocotyl flavonoids and membrane stability index (Table 1). In addition, the effect of foliar spraying of anti-stress compounds on leaf phenolic traits was significant,

whereas the other leaf phytochemical characteristics were not affected by the tested factors and their interaction (Table 1).

Table 1. Results of analysis of variance relevant to the effects of sucrose spraying, salicylic acid, SNP, and Bordeaux mixture on phytochemical content of radish.

Source of variance	df	Leaf total antioxidant capacity	Storage hypocotyl total antioxidant capacity	Storage hypocotyl total phenol content	Leaf total phenol content	Leaf total flavonoids	Storage hypocotyl total flavonoids	Membrane stability index percent (MSI)
Sucrose	1	0.71 ^{ns}	8.30 ^{ns}	0.0106 ^{ns}	0.1250 ^{ns}	1.793 ^{ns}	0.0035 *	2695.84 **
Anti-Stress	3	8.32 ^{ns}	35.30 ^{ns}	0.0256 ^{ns}	0.293 **	0.474 ^{ns}	0.0013 ^{ns}	1016.04 **
Sucrose × Anti-Stress	3	3.15 ^{ns}	21.60 ^{ns}	0.0112 ^{ns}	0.110 ^{ns}	0.602 ^{ns}	0.0041 *	1068.81 **
Error	16	13.832	18.769	0.0161	0.0459	0.266	0.00095	62.31
Coefficient of Variation		4.46	4.69	16.83	14.82	14.84	40.35	9.42

ns, *, ** not significant, or significant at p≤0.05 or p≤0.01, respectively, ANOVA.

Plants treated with plant anti-stress compounds had leaf total phenol content 30% higher than the control (Figure 1).

The ANOVA revealed a significant effect of interaction sucrose × anti-stress compounds on the storage hypocotyl flavonoids. This parameter was higher in plants sprayed with sucrose + Bordeaux mixture (0.125 µg Quercetin/mg extract) compared to plants treated with sucrose and salicylic or SNP (Figure 2).

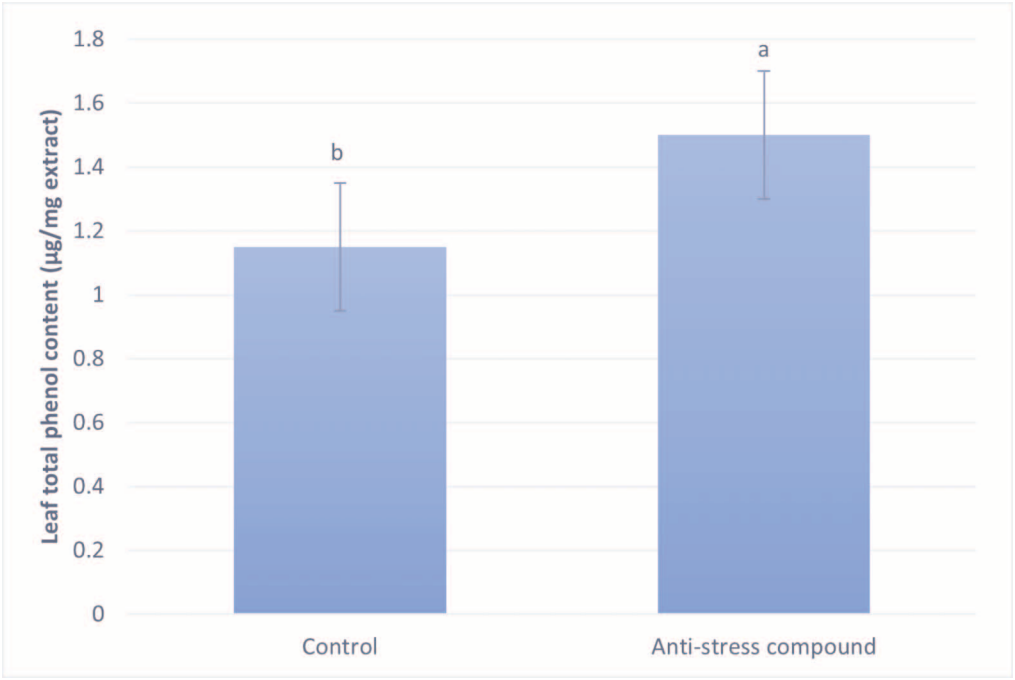

Figure 1. The effect of anti-stress compounds on radish leaf phenol content (n=3). Bars represent standard errors of the mean. Different letters indicate significant differences between treatments according to the Tukey test (p≤0.05).

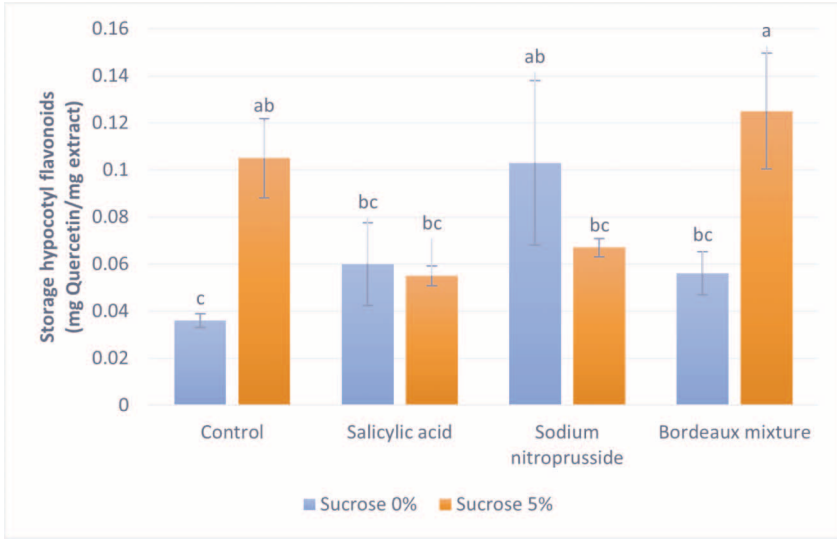

Figure 2. The effect of sucrose and anti-stress compounds on radish storage hypocotyl flavonoid content (n=3). Bars represent standard errors of the mean. Different letters indicate significant differences between treatments according to the Tukey test ($p \leq 0.05$).

The lowest MSI values were measured in the untreated plants and in those sprayed with salicylic acid alone (Figure 3).

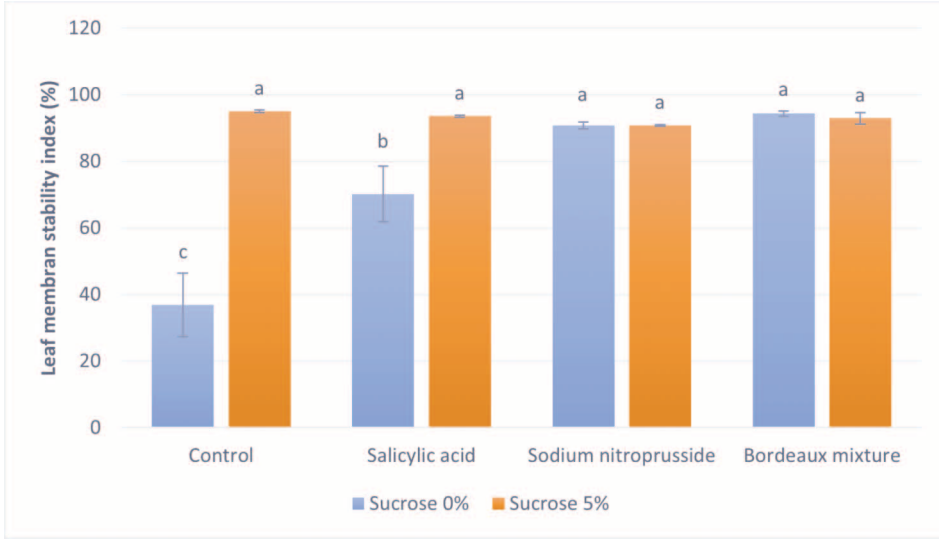

Figure 3. The effect of sucrose and plant growth promoting on radish leaf membrane stability index percentage (n=3). Bars represent standard errors of the mean. Different letters indicate significant differences between treatments according to the Tukey test ($p \leq 0.05$).

3.2. Photosynthesis pigments

The interaction effect of sucrose and anti-stress compounds foliar spraying significantly affected chlorophyll b, total chlorophyll and chlorophyll a/chlorophyll b (Table 2) The chlorophyll a and carotenoid content were not affected by the two experimental factors and their interaction (Table 2).

Plants sprayed with Bordeaux mixture + sucrose had the highest chlorophyll b content (0.928 mg/g FW) and total chlorophyll (2.976 mg/g FW) in the leaves which were 41.8% and 35.0% higher than control, respectively (Figures 4 and 5). No significant differences were found in these parameters between the other treatments (Figures 4 and 5). Plants sprayed with SNP+ sucrose had a chlorophyll a /chlorophyll b ratio significantly higher than control (Figure 6).

Table 2. Results of analysis of variance relevant to effects of spraying sucrose, salicylic acid, sodium nitroprusside, and Bordeaux mixture on chlorophyll content of Radish.

Source of variance	df	Chlorophyll a	Chlorophyll b	Carotenoids	Total chlorophyll	Chlorophyll a / Chlorophyll b
Sucrose	1	0.238 ^{ns}	0.108 *	0.083 ^{ns}	0.0784 ^{ns}	0.0419 ^{ns}
Anti-Stress	3	0.224 ^{ns}	0.0862 *	0.794 ^{ns}	0.7856 *	0.097 ^{ns}
Sucrose × Anti-Stress	3	0.094 ^{ns}	0.083*	0.312 ^{ns}	0.773 *	0.109 **
Error	16	0.224	0.02532	0.014	0.2047	0.0826
Coefficient of variation		33.67	29.26	40.67	24.07	10.34

ns, *, ** not significant, or significant at p≤0.05 or p≤0.01, respectively, ANOVA.

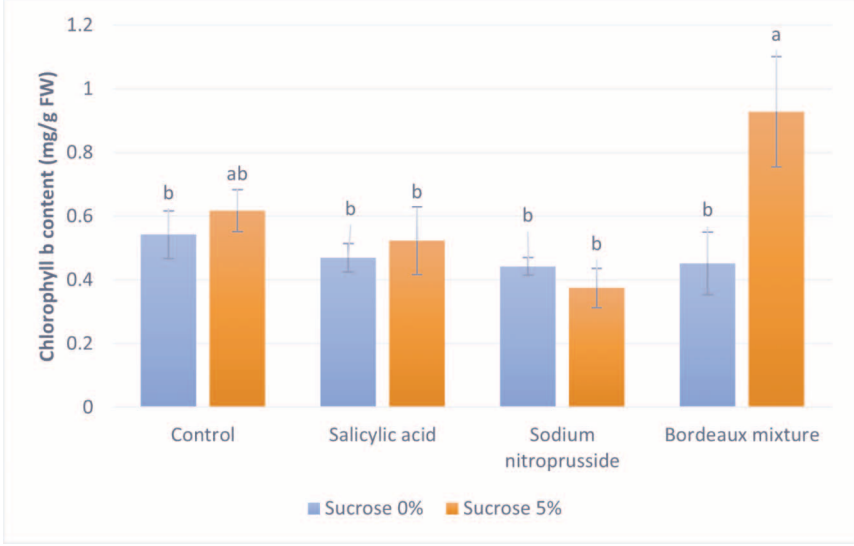

Figure 4. The effect of sucrose and anti-stress compounds on radish chlorophyll b content (n=3). Bars represent standard errors of the mean. Different letters indicate significant differences between treatments according to the Tukey test (p≤0.05).

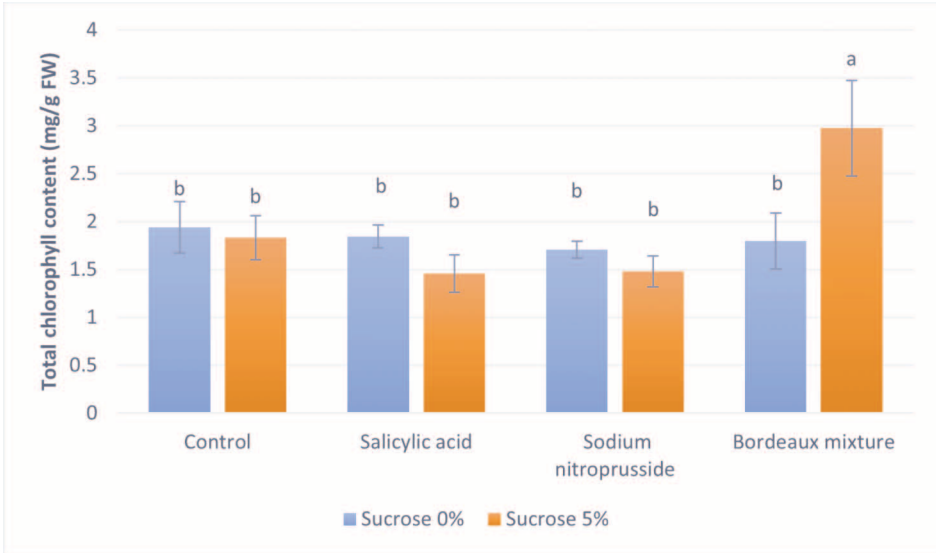

Figure 5. The effect of sucrose and anti-stress compounds on radish total chlorophyll content (n=3). Error bars represent standard errors of the mean. Letters above each bar indicate significant differences between treatments according to the Tukey test (p≤0.05).

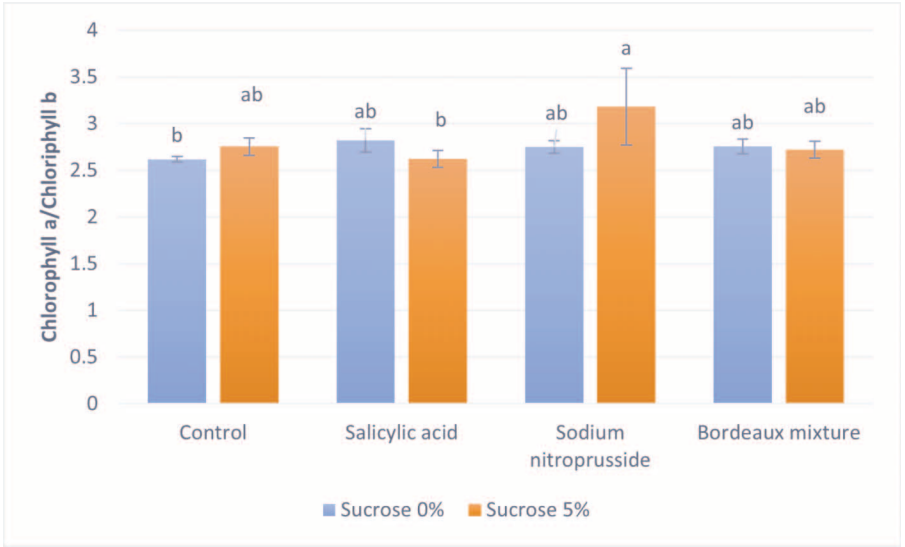

Figure 6. The effect of sucrose and anti-stress compounds on radish Chlorophyll a/ Chlorophyll b ratio (n=3). Error bars represent standard errors of the mean. Letters above each bar indicate significant differences between treatments according to the Tukey test ($p \leq 0.05$).

3.3. Carbohydrate, proline, and membrane peroxidation

The interaction between sucrose and anti-stress compounds significantly affected leaf total soluble carbohydrates, leaf lipid peroxidation, leaf proline the storage hypocotyl proline, and lipid peroxidation (Table 3).

Table 3. Results of analysis of variance relevant to effects of spraying sucrose and anti-stress compounds on sugar content, proline, and lipid peroxidation of radish.

Source of variance	df	Leaf soluble carbohydrates	Leaf lipid peroxidation (MDA)	Storage hypocotyl lipid peroxidation (MDA)	Storage hypocotyl Proline	Leaf proline
Sucrose	1	2730.18 **	0.0018 **	1.030 **	33.834*	91.90 *
Anti-Stress	3	482.43 *	0.0010*	0.692 **	17.83 *	255.33 **
Sucrose × Anti-Stress	3	817.68 **	0.0022 **	0.265 *	1.902*	121.29 **
Error	16	116.04	0.00027	0.0622	5.032	21.64
Coefficient of variation		20.75	24.07	24.68	44.31	30.69

ns, *, ** not significant, or significant at $p \leq 0.05$ or $p \leq 0.01$, respectively, ANOVA.

Leaf soluble carbohydrates content in plants sprayed with sucrose + Bordeaux mixture was significantly higher than all other treatments (65% higher than control, 62% than plants sprayed with only SNP, and 63% than plants treated with only Bordeaux mixture) (Figure 7).

The highest leaf lipid peroxidation occurred in plants treated with salicylic acid without the use of sucrose, whereas there was no significant difference in this parameter between the other treatments (Figure 8). Plants sprayed with Bordeaux mixture + sucrose and those treated only with SNP without sucrose had the lowest storage hypocotyl lipid peroxidation (Figure 9).

The highest leaf proline was detected in plants sprayed with sucrose+ Bordeaux mixture (Figure 10). Control plants (no sucrose) showed the highest proline concentration in the storage hypocotyl with the exception for control plants treated with sucrose and those sprayed only with salicylic acid (Figure 11).

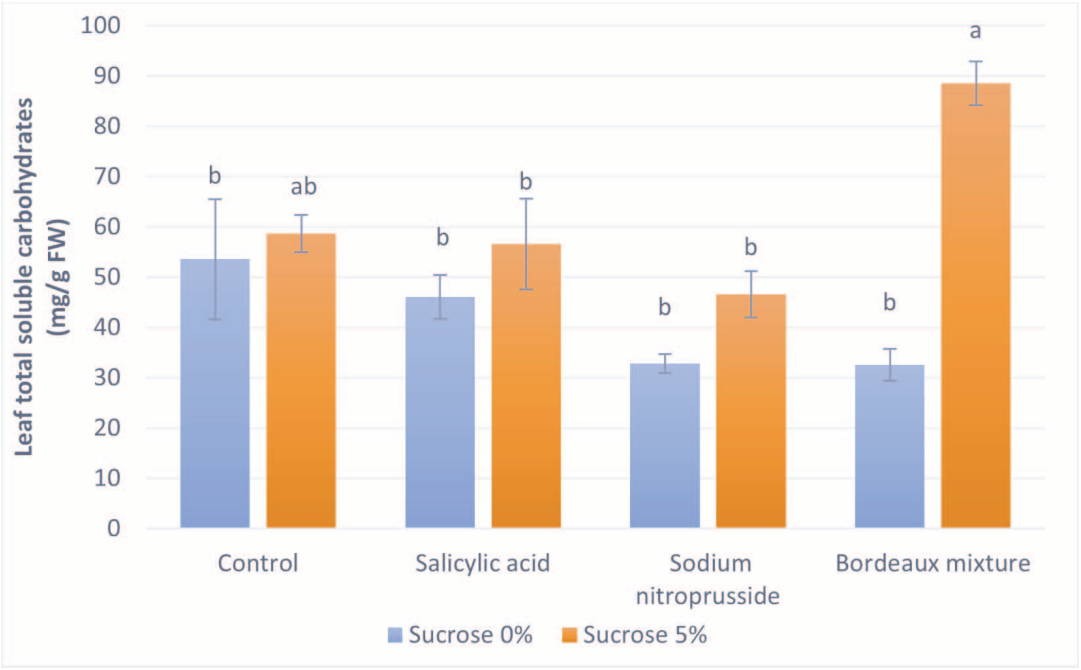

Figure 7. The effect of sucrose and anti-stress compounds on radish leaf total soluble carbohydrates content (n=3). Error bars represent standard errors of the mean. Letters above each bar indicate significant differences between treatments according to the Tukey test ($p \leq 0.05$).

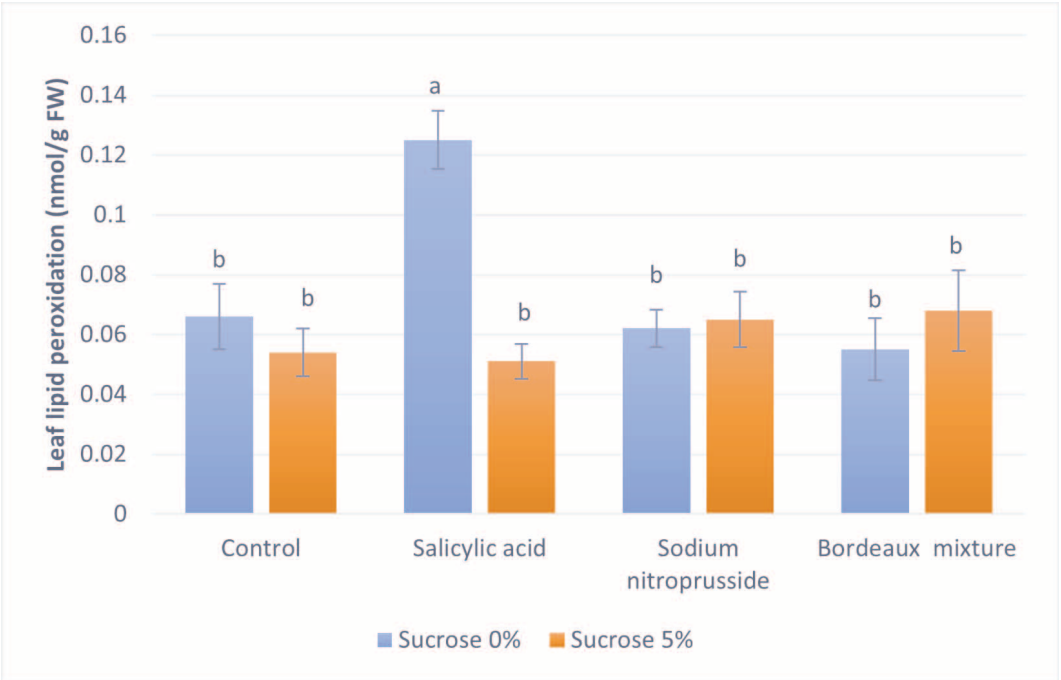

Figure 8. The effect of sucrose and anti-stress compounds on radish leaf lipid peroxidation (n=3). Error bars represent standard errors of the mean. Letters above each bar indicate significant differences between treatments according to the Tukey test ($p \leq 0.05$).

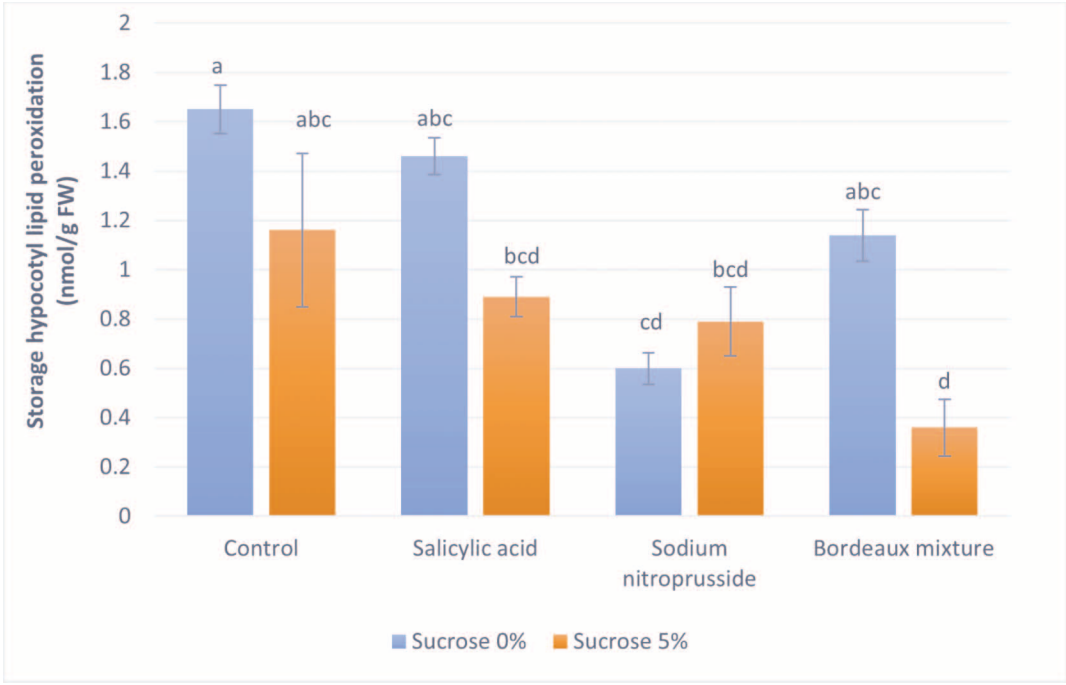

Figure 9. The effect of sucrose and anti-stress compounds on radish storage hypocotyl lipid peroxidation (n=3). Error bars represent standard errors of the mean. Letters above each bar indicate significant differences between treatments according to the Tukey test ($p \leq 0.05$).

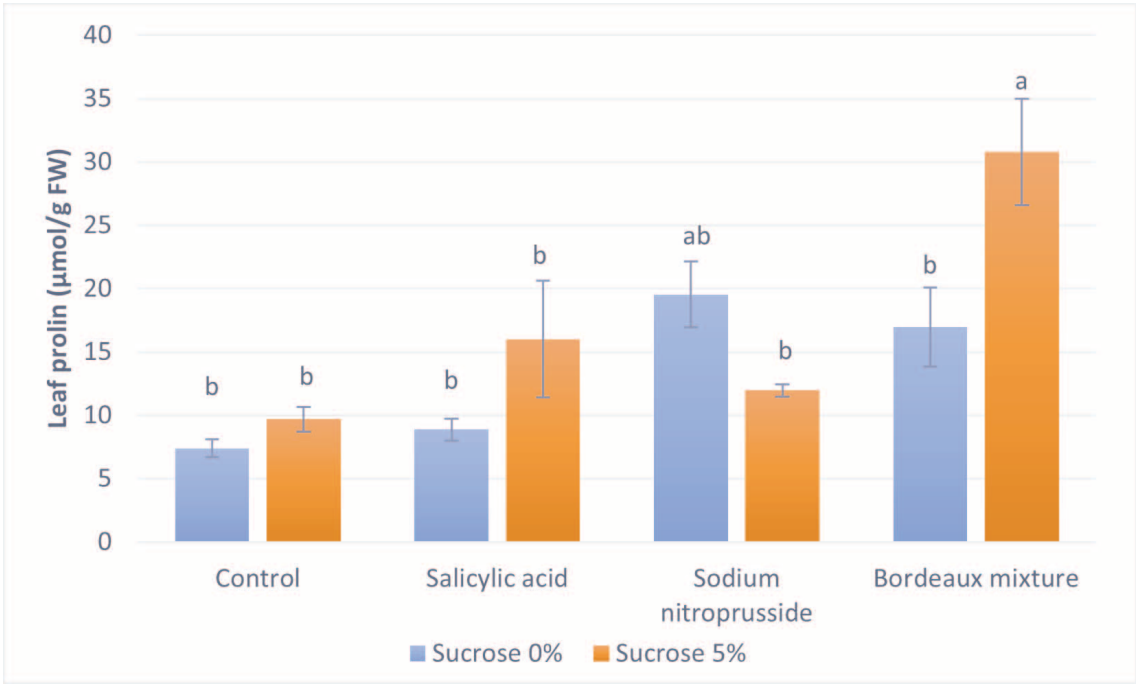

Figure 10. The effect of sucrose and anti-stress compounds on radish leaf proline content (n=3). Error bars represent standard errors of the mean. Letters above each bar indicate significant differences between treatments according to the Tukey test ($p \leq 0.05$).

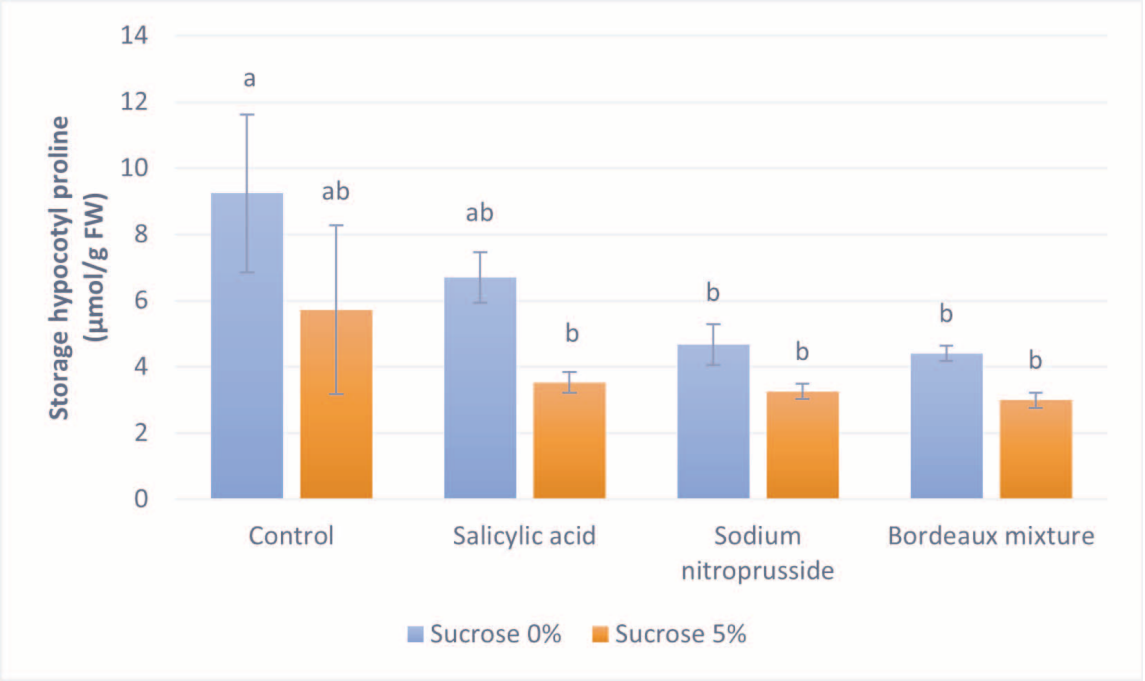

Figure 11. The effect of sucrose and anti-stress compounds on radish storage hypocotyl proline content (n=3). Error bars represent standard errors of the mean. Letters above each bar indicate significant differences between treatments according to the Tukey test (pP≤0.05).

The correlation between the investigated traits are shown in Table 4. Significant negative correlations were found between the leaf membrane stability percentage and storage hypocotyl lipid peroxidation (r=-0.79) between membrane stability and storage hypocotyl proline (r=-0.89). Moreover, chlorophyll b was positively correlated with leaf soluble carbohydrates (r=0.91)and leaf proline (r=0.65), whereas leaf proline was positively correlated with leaf flavonoid and storage hypocotyl flavonoid (r=0.66 and r=0.65, respectively). Hypocotyl lipid peroxidation was negatively a correlated with storage leaf and storage hypocotyl flavonoids (r=-0.74). Moreover, a positive correlation was detected between storage hypocotyl proline and leaf lipid peroxidation (r= 0.33), whereas leaf proline and storage hypocotyl lipid peroxidation resulted negatively correlated (r=-0.85). Furthermore, a negative significant correlation was found between storage hypocotyl lipid peroxidation and storage hypocotyl flavonoid (r=-0.74).

4. Discussion

All the treatments used in this experiment had a positive effect on membrane stability by inhibiting membrane electrolyte leakage in radish leaves. Since applying sucrose alone induced a high membrane stability, probably in the combined treatment of salicylic acid + sucrose, sucrose was the main factor inducing high membrane integrity by reducing electrolyte leakage, whereas salicylic acid did not play a significant role in this regard. Indeed, the use of sucrose increased membrane integrity in both control and treated samples. The highest increase was observed compared with the control without sucrose. In general, freezing tolerance of cells after sucrose treatment is achieved by a complex mechanism including significant changes in cell structure and accumulation of specific sugars and proteins (Jitsuyama et al., 2002). Physiologically, it is thought that sucrose stabilizes sensitive cellular components under stress conditions and acts as an osmotic protector (Korn et al., 2008).

The application of SNP and Bordeaux mixture, either alone or in combination with sucrose, induced an increase in the membrane stability index, which indicates the independent role of these compounds apart sucrose in determining this trait. The copper compound probably inhibited the ice forma-

Table 4. Correlation coefficient (Pearson's r) between physiological parameters in radish.

	Membrane stability index (MSI)	Total chlorophyll	Chlorophyll b	leaf soluble carbohydrates	Leaf lipid peroxidation	Storage hypocotyl lipid peroxidation	Storage hypocotyl proline	Leaf proline	Storage hypocotyl phenol	Leaf flavonoids	Storage hypocotyl flavonoids	Chlorophyll a
Membrane stability index (MSI)	1											
Total chlorophyll	0.05 ns	1										
Chlorophyll b	-0.05 ns	0.91 **	1									
Leaf soluble carbohydrates	-0.01 ns	0.76 *	0.91 **	1								
Leaf lipid peroxidation	-0.25 ns	0.69 *	-0.08 ns	-0.04 ns	1							
Storage hypocotyl lipid peroxidation	-0.79 *	-0.34 ns	-0.39 ns	-0.34 ns	0.35 *	1						
Storage hypocotyl proline	-0.89 *	-0.08 ns	-0.17 ns	-0.19 ns	0.33 *	0.86 **	1					
Leaf proline	-0.48 ns	0.67 *	0.65 **	0.48 ns	-0.25 ns	-0.85 **	-0.68 *	1				
Storage hypocotyl phenol	0.38	-0.04 ns	0.052 ns	0.28 *	0.19 ns	0.43 ns	0.48 ns	-0.60 *	1			
Leaf lavonoids	-0.54 *	0.57 *	0.62 **	0.47 ns	-0.14 ns	-0.74 *	-0.49 ns	0.66 *	-0.04 ns	1		
Storage hypocotyl flavonoids	-0.54 *	0.57 *	0.62 **	0.47 ns	-0.14 ns	-0.74 *	-0.49 ns	0.65 *	-0.04 ns	1	1	
Chlorophyll a	-0.35 ns	-0.27 ns	-0.40 ns	-0.21 ns	0.17 ns	-0.20 ns	-0.36 ns	-0.14 ns	0.24 ns	0.03 ns	0.03 ns	1

ns indicates no significant, * significant correlation at $p<0.05$, ** significant correlationat $p<0.01$

tion in plants by controlling the ice-nucleating bacteria and reducing the membrane electrolyte leakage. Many reports indicate the bactericidal effects of copper compounds (Pernezny et al., 2008). Many studies have focused on the use, efficacy, of the application of copper compounds to control bacterial diseases of Solanaceous crops (Graffin et al.,2017). Regarding SNP release of nitric oxide, it has been reported that this compound responds to oxidative stress conditions by increasing the activity of antioxidant enzymes (Rossatto et al., 2017; Singhal et al., 2021). Strawberry plants sprayed with mono potassium and SNP showed an increase in antioxidant enzyme activity, and the highest PAL enzyme activity was induced by the combined application of 3 g/L mono potassium and 600 μmol/L sodium nitroprusside (Saeedi et al., 2022).

Spraying SNP on plants under freezing stress caused an increase in antioxidant enzymes, reducing lipid peroxidation and preventing membrane electrolyte leakage. In this context, Zhao et al. (2009) reported that the increased proline accumulation in low-temperature stress conditions is caused by an increase in internal levels of nitric oxide. Since none of the treatments tested in our study could reduce the leaf membrane peroxidation in radish compared to the control, this suggests that at least these treatments could not effectively prevent the negative effects of freezing stress on the aerial part of the plant. The application of salicylic acid treatment alone increased lipid peroxidation compared to the control, which could be due to the production of ROS species such as H_20_2, H_2O_2-decomposing enzymes, and oxidative damage to proteins (Rao et al., 1997). In this regard, Cao et al. (2009) reported that transgenic Arabidopsis plants lacking salicylic acid were more resistant to moderate salt stress than the control.

Radish hypocotyl storage membrane peroxidation was strongly inhibited by the application of sucrose + Bordeaux mixture. Interestingly, storage hypocotyl flavonoid levels were also high by this treatment, and a strong and significant negative correlation was found between flavonoids and storage hypocotyl membrane peroxidation. Plants sprayed with sucrose + Bordeaux mixture had high flavonoid levels, low proline levels, and low membrane peroxidation in the hypocotyl storage. This suggests that the flavonoid was effective in preventing hypocotyl storage membrane peroxidation, and therefore, the need for proline biosynthesis in the storage hypocotyl or its transfer from the leaves declined. Proline synthesis through the glutamate pathway normally occurs in the cytosol and chloroplast, but is more intense in the meristem at the tips of stems and leaves (Meena, 2019). Thus, in plants treated with sucrose + Bordeaux mixture proline was accumulated in leaves as there was no need to export to the belowground organs. Conversely, in control plants characterized by the lowest flavonoid levels in hypocotyl storage, the need for hypocotyl storage proline increased. Therefore, the highest concentration of hypocotyl storage proline was measured in these plants. Although the control plants had a high proline content in their hypocotyl storage, it could not prevent the storage hypocotyl membrane peroxidation, and this indicates the marginal role of proline in maintaining cell membrane structure, but probably other factors such as the flavonoid level are more effective in this regard. Shomali et al., (2022) reported that flavonoids help alleviating temperature stresses by acting as antioxidants, stabilizing cellular membranes, and modulating stress-responsive pathways. In addition, flavonoids were proposed to play a role in freezing tolerance because these compounds participate in the protection of cell membranes and proteins against cold stress (Pawlikowska-Pawlęga et al., 2014). Wang et al. (2006) reported that the possible protective mechanism of flavonoids during freezing is related to the role of flavonoids in preventing membrane peroxidation due to their antioxidant activity. Moreover, previous studies reported that in Arabidopsis the flavonol content is related to leaf freezing tolerance and that the expression of some flavonoid biosynthesis genes is positively correlation with freezing tolerance (Schulz et al., 2015). Direct protection of membranes and proteins against freezing damage is another possible mechanism by which flavonoids can increase freezing tolerance, especially since flavonoids are localized in the vacuole, nucleus, and chloroplast, suggesting that these tissues may be protected by flavonoids during freezing (Agati et al., 2012). In addition to the increased hypocotyl storage tolerance to freezing stress, the combined treatment of the sucrose+ Bordeaux mixture increased the total chlorophyll and the leaf total soluble carbohydrates, and this suggests an improved photosynthetic activity.

Temperature affects the chloroplast membrane integrity irreversibly, disrupts the Rubisco enzyme activity, and affects the chlorophyll content (Taiz and Zeiger, 2018). The mode of action of sucrose in chlorophyll synthesis is probably related to the provision of carbon skeleton and the signalling effect of sucrose on the gene expression of chlorophyll synthesis. Zhou et al. (2016) showed that a delay in chlorophyll decomposition occurs in plants treated with sucrose in post-harvest treatment due to a reduction the activity of enzymes and the expression of genes related to chlorophyll degradation. The positive effect of copper on photosynthesis and chlorophyll content was also reported in wheat seedlings. The application of copper nanoparticles induced an increase in chlorophyll a, chlorophyll b and total chlorophyll, and a reduction of peroxidase enzyme activity (Essa et al., 2021).

Finally, it should be mentioned that treatments used in this experiment had more positive effects on the radish storage hypocotyls than leaves. In this context, other reports have indicated a defensive function of leaf organs under stress conditions in favour of storage organs. For example, Mogaddami et al. (2013) found that plants maintained more water in storage hypocotyl tissue than in leaves under drought stress. Rouhani et al. (2014) observed an increase in leaf sodium accumulation under water stress, and Galieni et al. (2018) noted limited growth and an increase in leaf thickness under drought stress. Additionally, Yildrim et al. (2022) found a greater increase in leaf cadmium compared to hypocotyl storage in plants grown under cadmium stress.

5. Conclusions

The results of this research demonstrate that most tested compounds showed potential to enhance freezing stress tolerance in radish storage hypocotyl. Notably, the combined treatment of sucrose + Bordeaux mixture proved most effective, increasing storage hypocotyl flavonoid, total chlorophyll, and leaf total soluble carbohydrates, while significantly reducing storage hypocotyl membrane peroxidation significantly. Since the combined treatment of sucrose + Bordeaux mixture has performed well in different investigated traits, it is a promising candidate for future research.

Conflict of interest

The authors indicate no conflict of interest in this work.

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