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RESEARCH ARTICLE

Effects of sodium nitroprusside on some physiological and histological responses of pepper plants exposed to salt stress

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ABSTRACT

Salinity exhibits many constraints in vegetable growing. Exogenous applications should be performed in growing in order to mitigate salinity damage. This study examined some physiological and histological properties of young pepper (*Capsicum annuum* L.) 'Çetinel' plants exposed to salt stress by applying different doses of sodium nitroprusside (SNP). Seedlings were transplanted into pots and salt application was started after 6 d. One day before salt stress, three different doses of SNP (12.5, 25.0 and 50.0 μM) were applied as a solution to the roots, excluding control and salt (NaCl) application. Then, 150 mM salinity was applied to all plants except the control. The experiment was terminated 35 d after the salinity treatment. At the end of the study, many histological and physiological parameters were evaluated. Plant growth, leaf relative water content (LRWC), total chlorophyll and carotenoid content of the leaves decreased in pepper plants under saline conditions, but SNP treatments increased the values. Membrane permeability in NaCl application increased by approximately 304% compared to control application. The SNP treatments increased xylem conduit diameter by 65.7% and epidermis thickness by 61.5% compared to salinity stress. When the results were evaluated, the values closest to the control in many parameters were recorded in the NaCl+SNP 25.0 μM application and it was found that it was effective in mitigating salinity stress damage. At the same time, 25.0 μM SNP application improved xylem vessels and cortical cells that may help plants acquire water and minerals more efficiently.

Key words: *Capsicum annuum*, cortex, salinity, sodium nitroprusside, xylem.

INTRODUCTION

Alterations in the plant's optimal environmental circumstances restrict its growth and development. These undesirable adverse conditions are called stress. Stress factors that cause many regressions resulting in low yield in the plant are classified in two ways as abiotic and biotic (Tuna and Eroğlu, 2017). Salt stress, which is one of the abiotic stresses, pollution of groundwater due to natural or artificial events, causes agriculture to be affected by low quality and high salt content irrigation waters. Salinity causes crop loss by causing osmotic stress in plants (Rao et al., 2016). Therefore, finding an effective way to reduce salinity damage is important for sustainable production.

Pepper (*Capsicum annuum* L.), belonging to the Solanaceae family, is a versatile plant that is widely grown in the world and can be consumed in different ways and for fresh consumption. Pepper is a moderately salt-sensitive vegetable (Velicevici et al., 2017), and when the salinity level in the water reaches electrical conductivity (EC) 4 dS m^{-1}, there is a significant decrease in yield (Semiz et al., 2014).

Some compounds that can be applied to plants before the onset of stress are accepted as an effective method for increasing tolerance, as they prepare them to cope with stress and alleviate the negative effects of salt on the plant (Tuna and Eroğlu, 2017). One of these compounds is the sodium nitroprusside (SNP). The SNP is a nitric oxide (NO) donor and NO is a hydrophobic gas molecule with a small size. Therefore, NO can easily move into hydrophilic parts of the cell, such as the cytoplasm, and easily pass through the lipid phase of membranes, acting as a biological signaling pathway between cells. It is thought to play an important role in the growth and development of plants from the seed to the flowering stage and in the ripening of fruits (Kovacs et al., 2015). When seedlings were exposed to NO therapy, antioxidant enzyme activity increased and membrane lipid peroxidation was reduced under salinity stress (Khoshbakht et al., 2018). It has been reported that NO forms defense mechanisms in response to stress by participating in plant defense with reactive oxygen species (ROS) and by sending signals to induce dead cells (Chavoushi et al., 2019). According to stress studies on plants, it was determined that there are two mechanisms of SNP. The first is that it acts as an antioxidant that scavenges ROS, second, the SNP indirectly helps plants by acting as a signaling molecule (Aras et al., 2020). Studies have been conducted in many plant species to determine the effectiveness of SNP under salt stress (Khoshbakht et al., 2018; Shams et al., 2019; Jabeen et al., 2021). Moreover, SNP provided early xylem formation in peach bud (Aras, 2022c) and xylem improvement (Aras, 2022b).

Although there are many studies on salt stress in pepper plants, studies are needed to determine the effects of SNP application on histological responses. For this reason, the effects of different SNP doses on the morphological, physiological, and histological properties were evaluated in young pepper plants exposed to salinity stress.

MATERIALS AND METHODS

The study was conducted in a controlled greenhouse (approximately 26-30 °C day and 18-22 °C at night, relative humidity 50-70%) at the Yozgat Bozok University Faculty of Agriculture. Pepper (*Capsicum annuum* L.) 'Çetinel', moderately salinity-resistant (Emirzeoğlu and Basak 2020), was planted in 2 L pots filled with peat:perlite mixture (1:1, v:v) in 2021. The experiment was designed according to the randomized plot design with three replicates and five plants per replicate. The first salinity treatment was started 6 d after planting (when the plants had 5-6 true leaves). One day before salinity stress, three different sodium nitroprusside (SNP) doses (12.5, 25.0 and 50.0 μM) were applied to plant rhizosphere as solution except control and salt (NaCl) treatment. The 150 mM salinity was applied to all plants except the control. Salinity stress was applied to the plant roots through each irrigation, and the experiment was terminated 35 d after the first salinity treatment application. The irrigation was performed once 2 d as performing of 20% drainage depending on plant growth status and excess solution was allowed to drain from the pot. The electrical conductivity (EC) values of control and salinity were 0.64 and 11.9 dS m^{-1}, respectively.

Measurements

At the end of the experiment, the part of the plants from the root collar to the growth tip was measured in cm with the help of a tape measure, and the stem diameter was measured in mm using a digital caliper.

The procedure of leaf relative water content (LRWC) based on Smart and Bingham (1974) was used to assess relative water content. For this, individual fresh weights (FW) of young leaves, which have completed their development, were taken at each replicate. Then, leaves were floated in distilled water for 6 h referenced as turgor weights (TW) at the end of the appropriate period. Dry weights (DW) of leaf samples, which were kept at 72 °C for 48 h, were taken. The calculation was made using the following formula:

$$LRWC (\%) = [(FW - DW)/(TW - DW)] \times 100$$

Membrane permeability (MP) was determined using an EC meter. Leaf samples (1 cm × 1 cm) were immersed in 10 mL distilled water and incubated for 24 h on a shaker at room temperature (25 °C). The first EC was recorded (EC1). Afterwards, the samples were autoclaved at 120 °C for 20 min and the second measurement was made (EC2). Membrane permeability (EC1/EC2×100) was determined according to Lutts et al. (1996).

To determine the amount of chlorophyll (*a*, *b* and *a*+*b*) and carotenoids, 0.25 g samples were taken from leaves of three plants in each experimental unit and filtered after being extracted with 80% acetone. The absorbance of the extract solution was read in the spectrophotometer at 645 and 663 nm; 450 nm reading was taken for carotenoid determination. The data obtained were calculated according to the formulas specified in Güneş et al. (2007).

To determine the amount of the malondialdehyde (MDA), samples of leaves (0.5 g) were homogenized with 5 mL 0.1% trichloroacetic acid (TCA) and centrifuged in for 5 min and at 15000 rpm. Aliquots of 1 mL each supernatant were mixed with 5 mL 0.5% thiobarbituric acid (TBA) in 20% TCA. The microcentrifuge tubes were incubated at 95 °C for 30 min and after cooling they were centrifuged for 5 min at 6000 rpm. The absorbance at 440, 532, and 600 nm was read on a UV-Vis spectrophotometer (Madhavo Rao and Sresty, 2000).

Histological studies

Leaf samples were stored in 70% ethanol. Freehand cross sections of leaf midrib were used for microscopic evaluation. The midrib sections reacted with special reagents: Toluidine blue O and phloroglucinol-hydrochloric acid (Aras et al., 2022). The stained samples were placed on the slide, visualized using a light microscope (Olympus CX21, Olympus Corporation of the Americas, Center Valley, Pennsylvania, USA) attached to a digital camera (Camera 5) at 4X, 10X, and 40X magnifications. Cortical cell diameter (μm) (10X), thickness (μm) (4X) of cortex, epidermis and midrib and xylem parameters were measured.

Statistical evaluation of the obtained data was performed using the SPSS Statistics 20.0 (IBM, Armonk, New York, USA). Duncan multiple range test (Duncan multiple comparison test) was applied to determine the differences between the applications. In the statistical evaluation of the results, the significance level between the differences was determined as $P < 0.05$. The correlation between applied and examined parameters was determined by principal component analysis (PCA) using XLSTAT software and agglomerative hierarchical clustering (AHC) classifies all treatments according to investigated parameters.

RESULTS

In this study, pepper plants were exposed to salinity at 150 mM for 35 d at the vegetative stage and relative damage to the indices of stress and the role of exogenously supplied SNP was evaluated. Salinity stress limited the growth of pepper plants, but the effect of SNP applications on plant height and stem diameter measurements was found to be significant (Table 1). Plant height values were recorded as 58.0 cm in the control application and 32.0 cm in the salinity treatment. The highest plant height increased in SNP applications compared to salt application was measured as 46.0 cm in 25.0 μM SNP application. The stem diameter measurements were 6.89 mm in the control application and 3.64 mm in the salinity treatment. Application of 25.0 μM SNP (5.08) significantly increased stem diameter compared to the application of salt.

Table 1. Effect of sodium nitroprusside (SNP) applications on plant height and stem diameter measurements in pepper. Duncan's multiple range test determines the degree of separation within a column. The significance level was $p < 0.05$.

Treatments	Plant height	Stem diameter
	cm	mm
Control	58.00^{a}	6.89^{a}
NaCl	32.00^{d}	3.64^{c}
NaCl+SNP 12.5 μM	34.67^{cd}	3.77^{c}
NaCl+SNP 25.0 μM	46.00^{b}	5.08^{b}
NaCl+SNP 50.0 μM	36.67^{c}	3.96^{c}

It was observed that the leaf relative water content (LRWC) decreased in pepper plants under saline conditions. Although the LRWC was 86.54% in the control group, it decreased to 56.06% in the NaCl plants. The LRWC, which decreased with the effect of salt, increased with the effect of externally applied SNP. The highest increase was recorded with 81.21% by 25.0 µM SNP application (Table 2).

As shown in Table 2, the membrane permeability (MP) value increased with the effect of salt, but SNP application was effective in decreasing this value. While MP was 28.76% in the control application, it increased to 87.37% compared with the NaCl treatment only, and an increase of about 304% was observed. The lowest MP was recorded in the control, and the closest value to the control was measured as 59.23 in the 25.0 µM SNP application.

Changes in the MDA content are shown in Table 2. The fact that the MDA content reaches higher levels in the NaCl application than in the control indicates that the cell membranes are damaged by the effect of salinity. The MDA, which increased because of salt stress, decreased with SNP application.

Chlorophyll *a*, *b*, *a+b* and carotenoid concentrations are given in Table 3, and significant differences were found between applications. The highest chlorophyll *a* value was recorded in 25.0 µM SNP application, and chlorophyll *b* value was recorded in 12.5 µM SNP application. Chlorophyll *a+b* and carotenoid values were found to be higher in all SNP applications compared with NaCl application.

Table 2. Effect of sodium nitroprusside (SNP) applications on membrane permeability (MP), leaf relative water content (LRWC) and malondialdehyde (MDA) measurements in pepper. Duncan's multiple range test determines the degree of separation within a column. The significance level was $p < 0.05$.

Treatments	MP	LRWC	MDA
	%	%	µmol g^{-1} fw
Control	28.76^{c}	86.54^{a}	0.982^{c}
NaCl	87.37^{a}	56.06^{d}	3.015^{a}
NaCl+SNP 12.5 µM	78.53^{a}	73.48^{c}	2.311^{ab}
NaCl+SNP 25.0 µM	59.23^{b}	81.21^{b}	1.863^{b}
NaCl+SNP 50.0 µM	80.27^{a}	72.26^{c}	2.734^{ab}

Table 3. Effect of sodium nitroprusside (SNP) applications on chlorophyll and carotenoid concentrations in pepper. Duncan's multiple range test determines the degree of separation within a column. The significance level was $p < 0.05$.

Treatments	Chlorophyll *a*	Chlorophyll *b*	Chlorophyll *a+b*	Carotenoid
	µg g^{-1} fw			
Control	35.28^{ab}	33.20^{b}	68.48^{a}	3.131^{a}
NaCl	34.28^{ba}	28.72^{c}	63.01^{b}	2.505^{e}
NaCl+SNP 12.5 µM	33.75^{c}	38.92^{a}	72.67^{a}	2.647^{d}
NaCl+SNP 25.0 µM	35.81^{a}	33.10^{b}	68.91^{a}	2.879^{b}
NaCl+SNP 50.0 µM	34.81^{abc}	35.93^{ab}	70.74^{a}	2.739^{c}

After SNP applications, transverse sections of pepper leaf midribs were taken and stained with toluidine blue O and acid phloroglucinol to determine the basic anatomical structure (Figure 1). There was nonsignificant difference between applications in cortex thickness measurements (Table 4). However, the highest values in cortical cell diameter values were recorded in control and 25.0 µM SNP applications, and the difference between applications were found to be significant. The effect of applications on the number of cortex cells was significant, and the highest values were recorded in NaCl and 12.5 µM SNP applications.

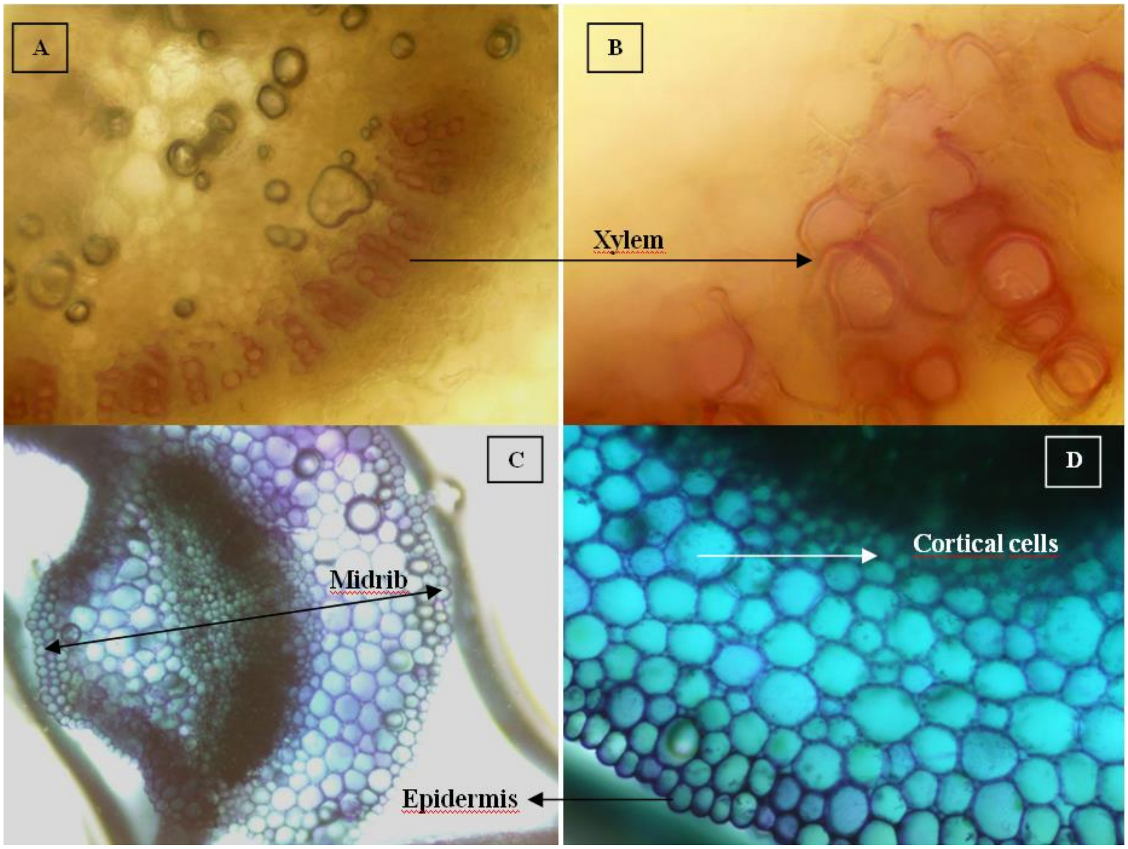

Figure 1. Histological characterization of pepper leaf midrib section images: Phloroglucinol dye showing xylem (10X and 40X, respectively) (A-B), toluidine blue dye showing midrib (4X) (C), toluidine blue dye showing cortical cells and epidermis (40X) (B).

Table 4. Effect of sodium nitroprusside (SNP) applications on cortical cell diameter, cortex thickness and cortical cell number of pepper plant. Duncan's multiple range test determines the degree of separation within a column. The significance level was $p < 0.05$. ns: nonsignificant.

Treatments	Cortical cell diameter	Cortex thickness	Cortical cell number
	µm		
Control	59.14[a]	385.00[ns]	6.54[b]
NaCl	40.85[b]	317.50	7.79[a]
NaCl+SNP 12.5 µM	45.32[b]	349.50	7.72[a]
NaCl+SNP 25.0 µM	57.63[a]	353.00	6.13[b]
NaCl+SNP 50.0 µM	47.27[b]	335.00	7.04[ab]

The effect of SNP applications on midrib diameter measurements was found significant and the highest value was recorded in 25.0 µM SNP application (Table 5). However, xylem conduits diameter and epidermis thickness were significantly affected by salt stress. The SNP applications increased in both parameters compared to NaCl stress, and the highest values were recorded as 22.82 and 40.30 µm, respectively, in 25.0 µM SNP application (Table 5).

Fifteen data variables collected from plants subjected to five different treatments (control, NaCl, NaCl+SNP 12.5 µM, NaCl+SNP 25.0 µM and NaCl+SNP 50.0 µM) were evaluated using principal component analysis (PCA) (Figure 2). The PCA was scattered applications in all three quarters of the biplot.

The axes represented 75.10% (F1) and 15.93% (F2), showing 91.04% (F1+F2) of the total variation. The PC1 demostrated most of the parameters examined and showed strong positive correlations. However, MDA, MP and cortical cell number parameters were negatively correlated. The proximity of the applications to each other was determined in line with the parameters examined using agglomerative hierarchical clustering (AHC) (Figure 3). According to the dendrogram, the applications were grouped under the number of two clusters.

Table 5. Effect of sodium nitroprusside (SNP) applications on midrib diameter, xylem conduits diameter and epidermis thickness of pepper plant. Duncan's multiple range test determines the degree of separation within a column. The significance level was $p < 0.05$.

Treatments	Midrib diameter	Xylem conduits diameter	Epidermis thickness
	µm		
Control	1246[ab]	21.96[a]	35.79[b]
NaCl	861[c]	13.77[c]	24.96[e]
NaCl+SNP 12.5 µM	1092[b]	17.42[b]	29.12[d]
NaCl+SNP 25.0 µM	1289[a]	22.82[a]	40.30[a]
NaCl+SNP 50.0 µM	1093[b]	18.72[b]	32.20[c]

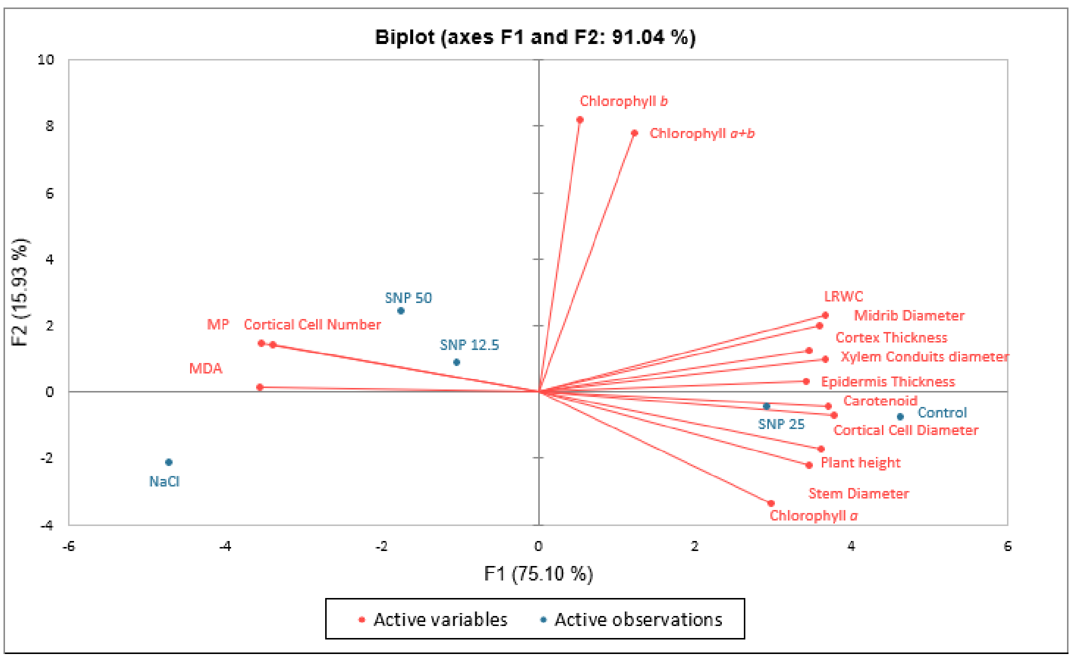

Figure 2. Principle component analysis of physiological and histological traits for different treatments. SNP: Sodium nitroprusside; MP: membrane permeability; MDA: malondialdehyde; LRWC: leaf relative water content; SNP 12.5: NaCl+12.50 µM SNP; SNP 25: NaCl+25.0 µM SNP; SNP 50: NaCl+50.0 µM SNP.

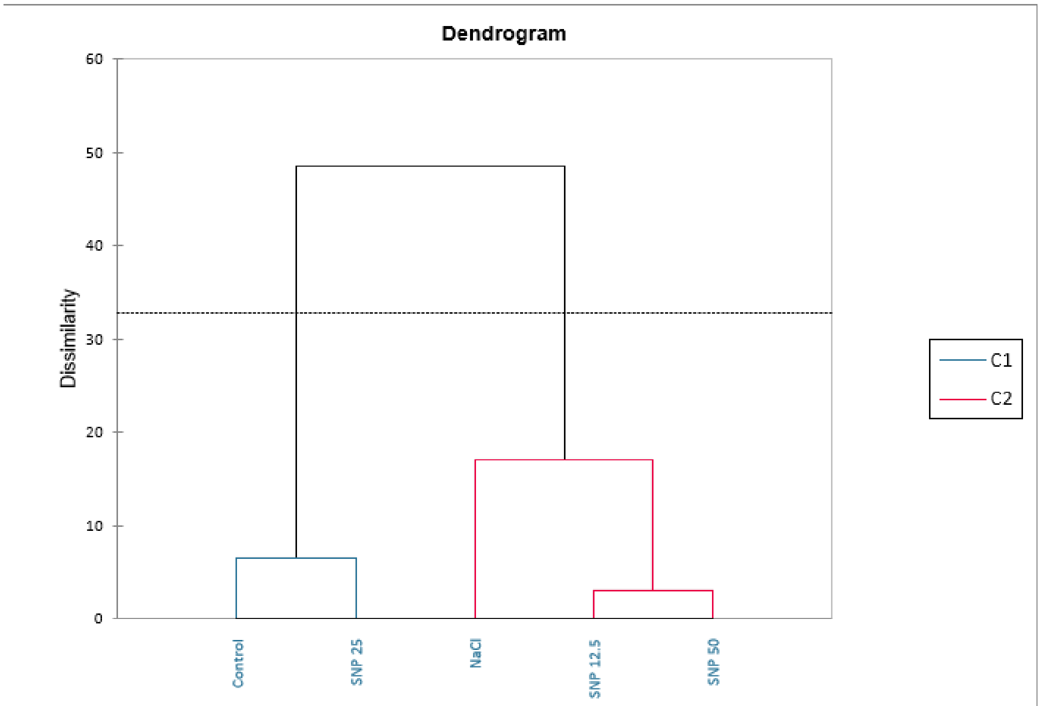

Figure 3. Dendrogram showing similarity between applications. SNP: Sodium nitroprusside; SNP 12.5: NaCl+12.50 µM SNP; SNP 25: NaCl+25.0 µM SNP; SNP 50: NaCl+50.0 µM SNP.

DISCUSSION

Different genotypes of the same species may have different degrees of salt tolerance. Emirzeoğlu and Basak (2020) screened the salinity resistance levels of different pepper genotypes and reported that 'Çetinel' used in the experiment was moderately salinity-resistant, scaled as 3.25 between 0-5 scale according to Emirzeoğlu and Basak (2020). One of the first observed effects of salt stress is a decrease in the growth rate of plants (Salih et al., 2022; Teykin et al., 2022). It has been determined that while pepper plants are exposed to salt stress, plant height and stem diameter values significantly decrease, while SNP applications increase these values.

The LRWC is a measurement reflecting the metabolic activity in tissues by indicating the physiological water status of plants and is frequently used in stress studies (Khoshbakht et al., 2018). In many studies, it has been reported that salt stress causes a significant decrease in LRWC values; however, SNP application increases the proportional water content of leaves (Shams et al., 2019). The LRWC results in the study support previous studies.

Membrane permeability (MP) another investigated parameter can be defined as an ion imbalance that develops due to intracellular and extracellular osmotic incompatibility, especially in plants under salt stress. With the increase in the EC value, some ions, especially Ca and K, from intracellular electrolytes, come out of the plant. This situation causes Na/K and Na/Ca imbalances in the cell and irregularities in the general ion balance (Chakraborty et al., 2018). Tuna and Eroğlu (2017) reported that NaCl+100 µM SNP they applied to the pepper plant reduced the EC value by around 10% compared with the NaCl application. In our study, the maximum effect of SNP on the improvement of the MP value, which increased approximately three times with the effect of salinity, was determined at 25.0 µM, and it decreased by 32% compared to the NaCl application.

The formation of ROS is a general response of plants to stress conditions. These reactive oxygen species oxidize the lipids in the membranes of plant cells and cause the membrane structures to deteriorate. As a result, the membrane selective permeability property is lost or decreased (Chrysargyris et al., 2018). The MDA, which emerges as an intermediate in the oxidation reactions of cell membrane lipids, is important in determining how much damage is caused by stress in salt stress studies (Zeeshan et al., 2020). In this study, it was determined that SNP applications caused a decrease in MDA content in general and this decrease was 38% in 25 µM SNP application. Shams et al. (2019) reported that the application of 150 µM SNP in pepper exposed to 150 mM NaCl reduced MDA by 54%.

Photosynthetic activity and leaf chlorophyll content of pepper are adversely affected under salt stress. In a study, it was reported that there was a significant decrease in chlorophyll *a* and *b* content in soybean plants exposed to salt stress compared to the control application; however, exogenous SNP application under salt stress increased the total chlorophyll content compared to NaCl application (Jabeen et al., 2021). Similar results were obtained in this study, and it was also found that salt stress decreased carotenoid content, and SNP applications were effective in mitigating this negative effect.

Leaves are the most important organs that play a role in the continuity of plant life. Leaf expansion plays important roles in photosynthesis affected by cortical cell division and expansion (Aras, 2022a). Salt stress negatively affects cell division and expansion, which are necessary for developing the leaf (Robin et al., 2016). In this study, cortex cells of leaf midrib were observed. Dolatabadian et al. (2011) reported that the cortex thickness decreased in soybean plants exposed to salt stress. As a result of the cortex measurements made in the study, similar results were obtained, and it was determined that 25.0 µM SNP application increased the cortical cell diameter parameter and caused a decrease in the number of cortical cells. This leads to the conclusion that it encourages the formation of a small number of larger cells. Aras et al. (2021) in their study, found that plants with large cortical cells had more chlorophyll and chlorophyll precursors compared to plants with smaller cortical cells under conditions of Ca deficiency.

Midrib is an important part of the leaves that controls water and mineral transportation. Thickness of midrib diameter is associated with wide xylem, phloem, and cortex (Aras et al., 2022). In this study, the highest midrib diameter value was obtained in 25.0 µM SNP application. The increment in midrib diameter may be related to the increase in xylem conduits diameter. Xylem is important vascular bundle that transports water and minerals in higher plants (Aras, 2021). Xylem is composed of lignin (Fernández-Pérez et al., 2015) and lignin is a product of phenylpropanoid pathway (Volpi e Silva et al., 2019) thus, improvement in xylem conduits takes a pivotal role in water and mineral transport. Salt stress causes changes in the lignification of the xylem structure (Dolatabadian et al., 2011). Sánchez-Aguayo et al. (2004) reported that the number of lignified xylem cells increased in tomato plants under salt stress and this response gradually decreased from the root to the leaves. The SNP treatments increased xylem conduit diameter. The NO triggers xylem formation through programmed cell death (Yu et al., 2014). Some studies showed that NO is involved in xylem formation in buds and xylem improvement in leaves (Aras, 2022b; 2022c). In the current study we examined xylem conduit diameter of leaf midrib. Xylem conduits diameter and epidermis thickness measurements determined that salt stress caused a negative effect, but SNP applications were effective in reducing this negative effect. It has been reported that the epidermis plays a protective role against stress factors (Silva et al., 2021). The highest values in xylem conduits and epidermis thickness parameters were recorded in 25.0 µM SNP application.

It has been shown by PCA analysis that SNP doses applied to pepper plants exposed to salt stress have different effects. The PCA clearly indicated that 25.0 µM SNP application was superior to other applications based on its positive effects on effective in reducing salt stress. The treatments were divided into two categories by cluster analysis and 25.0 µM SNP was found in the same side of control plants. Sivakumar et al. (2020) attempted find the best screening method for salt tolerance in tomato genotypes and showed that the PCA technique can be used as a suitable method for selection and distinction of tomato germplasm.

CONCLUSIONS

The current study proved that the application of sodium nitroprusside (SNP) is one of the appropriate treatments to increase salt tolerance in plants (in young pepper plants), regulating many physiological and histological characteristics to reduce the negative effect of stress in plant cells. The application of 25.0 µM SNP improved xylem vessels and cortical cells that may help plants acquire water and minerals more efficiently. The current paper suggests that 25.0 µM SNP dose is suitable for application in pepper plants under salinity.

Author contribution
All roles were performed by the author.

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