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Full Length Article

Physiological and Biochemical Responses of Pepper (*Capsicum annuum*) Leaves to Salt Stress

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Abstract

The mechanisms of salt tolerance in pepper plants are still being studied. The effects of salinity intensity and duration of exposure on pepper (*Capsicum annuum* L.) growth, photosynthetic pigments, osmolyte accumulation (proline), oxidative stress markers, and antioxidant compounds were studied. The results showed that 150 m*M* NaCl stress inhibited pepper root growth significantly. Proline levels increased with salt stress intensity and duration. Chlorophyll content decreased by the impact of 150 m*M* NaCl stress. Long-term salinity stress (9 days) increased carotenoids content and extended salinity stress increased anthocyanins. Furthermore, when stressed plants were exposed to 150 m*M* NaCl for an extended period of time, the hydrogen peroxide content and lipid peroxidation (malondialdehyde) level increased. Total phenolic and total flavonoid contents, on the other hand, increased with exposure time. As a result, it is clear that *C. annuum* tolerated salt intensity and duration stress by increasing proline content for osmatic adjustment and reactive oxygen species quenching, variable antioxidants accumulation protecting the photosynthesis, membranes and other macromolecules, resulting in the reduction of oxidative stress caused by salinity in pepper. Other parameters, such as phytohormones and polyamines, had to be studied under the same stress circumstances in order to visualize their roles and crosstalk between all salt tolerance traits.

Keywords: Antioxidants; Compatible solute; Photosynthetic pigments; Plant growth; Oxidative stress markers; Salinity

Introduction

Plants are subjected to osmotic and ionic stresses as a result of salinity. Furthermore, these can trigger secondary stressors such as oxidative stress (Gupta and Huang 2014). Taken together, these stressors limit absorption and cause a large outflow of water and ions in plant cells, causing water and nutritional imbalances. In addition, salinity stress leads to tissue accumulation of Na^+, Cl^- and the generation of reactive oxygen species (ROS), which ultimately limit crop growth, yield and production of economically important crops such as grains and vegetables (Bojórquez-Quintal *et al*. 2014; Gupta and Huang 2014; Hasanuzzaman *et al.* 2021; Sachdev *et al*. 2021). When the electric conductivity of the soil solution reaches 4 dS m^{-1} or more, the soil is deemed salty. This threshold adversely affects crop productivity and quality. Moreover, most plant species are susceptible to salinity even if electrical conductivity is <4 dS m^{-1}. Pepper is moderately sensitive to salt stress greater than 1.5 dS·m^{-1} (Chinnusamy *et al*. 2005). To ameliorate the salt-induced damage, a combination of stress tolerance strategies simultaneously took place by *C. annuum* after exposure to salinity (Bojórquez-Quintal *et al*. 2014; López-Serrano *et al*. 2021). However, *C. annuum* physiological and metabolic responses depended on the genotype, growth stage and salinity level (De Pascale *et al*. 2003; Zamljen *et al*. 2022).

Plants subjected to salinity stress have numerous strategies to mitigate the detrimental impacts, including ion homeostasis, compatible solutes, antioxidant modulation, polyamines and phytohormones (Maršić *et al*. 2021). Plant osmotic stress is frequently coupled with ion accumulation, which produces nutritional imbalance and particular ion impacts. Meanwhile, ROS formed in plant cells as a result of salinity frequently damage biological membranes, proteins, and nucleic acids (Guo *et al*. 2020; Butt *et al*. 2021; Hasanuzzaman *et al*. 2021). Environmental stresses adversely affect *C. annuum* growth and the metabolic processes (Niu *et al*. 2010; Carvalho Lemos *et al*. 2019; Butt *et al*. 2021; Zamljen *et al*. 2022). In this sight, pepper has economic importance because it is grown for medical use, coloring agent, fresh consumption, as a spice, antioxidants source, and as an ornamental plant (Palevitch and Craker 1996; Salehi *et al*. 2018).

Pepper (*Capsicum annuum* L.) is a highly valued crop with high economic and medicinal importance. According to the International Pepper Community (IPC), global pepper production in 2022 reached 535,000 metric tons, with Vietnam, Brazil, Indonesia and India being the top producing countries. The IPC also reported that the global trade value of

pepper was approximately $3.1 billion in 2020, with the European Union being the largest importer. In addition to its economic value, pepper has also been used for centuries in traditional medicine for its potential health benefits. Studies suggest that phytochemicals found in pepper may have anti-inflammatory, antioxidant, and antimicrobial properties, among others (Takooree *et al*. 2019; Amaechi *et al*. 2021).

Salinity stress is a serious debacle for growth of plants, including pepper. Herein the ability of *C. annuum* to cope with high salinity levels, as well as the mechanisms of biochemical and physiological tolerance that may be enrolled, remain to be investigated (López-Serrano *et al*. 2021). The objective of this study was to identify *C. annuum* salt tolerance ability and the morpho-physiological and biochemical mechanisms involved in tolerance to induced salinity.

Materials and Methods

Soil preparation, experiment setup and plant growth condition

Soil used in the experiments consisted of vermiculite, peat moss, and perlite (1:2:1 ratio, respectively). The cultured pots with 2 weeks old plants were grown under specified conditions in the growth chamber with 80 µmol m^{-2} s^{-1} light for 14 h and temperature of 21°C (day) and 10°C (night), while relative humidity was 55–60%.

Salinity stress treatment

Two-week-old chili paper (*Capsicum annuum*) plants were irrigated three times per week with NaCl solution (150 m*M*; 15.42 dS m^{-1}) for up to 9 days. In parallel, controlled samples irrigate tap water under controlled growth conditions. Samples were collected at 6 and 9 days in the growth chamber (Fig. 1).

Growth parameters analysis

Seedlings root and shoot length were measured on two salinity stress periods after 6 and 9 days of exposure.

Quantification of photosynthetic pigments

Extraction and quantification of chlorophylls were carried out in accordance with Porra (2002). Leaf samples (20 mg) were lysed in 1 mL cold acetone (80%). After 1 h dark incubation, the extract was centrifuged at 4°C for 10 min and 13000 rpm. The absorbance of supernatant was measured at 646.6 and 663.6 nm. Sims and Gamon (2002) method was used to quantify anthocyanins and carotenoids. Anthocyanins was extracted by methanol:HCl:water (90:1:1v/v/v) solvent mixture while carotenoids were extracted by acetone-Tris buffer (pH 7.8, 80:20v/v) and the supernatant collected after centrifugation at 4°C for 10 min and 13000 rpm then was read at 470 and 529 nm.

Measurements of leaf free proline content

Frozen leaf samples (1 g) lysed in 10 mL sulphosalicylic acid (3% w/v). The extract was centrifuged 4°C for 10 min and 13000 rpm. The reaction mixture consisted of supernatant, glacial acetic acid, and ninhydrin reagent in a 1:1:1 ratio mixed gently. The mixture was boiled for 1 h at 100°C then cooled for 10 min on ice. The reaction mixture was mixed with toluene followed by vigorous stirring for chromophore extraction in the supernatant phase. The reaction was measured at 520 nm against toluene as blank. Proline content was calculated using a calibration curve and expressed as µg proline mg^{-1} fresh weight (Bates *et al*. 1973).

Hydrogen peroxide (H_2O_2) detection

Velikova *et al*. (2000) method was used to determine H_2O_2 content. A 100 mg of plant materials lysed on ice with trichloroacetic acid (TCA, 0.1% w/v). The extract was centrifuged at 4°C for 15 min and 15,000 g. the reaction mixture consisted of supernatant, potassium phosphate buffer (10 m*M*, pH 7.0) and 1 M KI in a 1:1:2 ratio mixed gently. The reaction was measured at 390 nm. Known H_2O_2 concentrations were used to create a standard curve, which was then used to determine the H_2O_2 content.

Lipid peroxidation assay

Hodges *et al*. (1999) used to detect malondialdehyde (MDA) level that was considered as an end product of lipid peroxidation. Frozen leaf samples (50 mg) were lysed in ice cold 1 mL ethanol (80%) then the extract was centrifuged at 4°C for 20 min at 16,000×*g*. MDA detection was started by mixing the supernatant (0.5 mL) with 500 µL trichloroacetic acid (TCA, 20% w/v) containing thiobarbituric acid (TBA, 0.65% w/v). An ice bath was used to quickly cool the reaction after it was incubated at 95°C for 30 min. Following centrifugation at 10,000×*g* for 10 min, the reaction absorbance was measured at 532 and 600 nm. The MDA content was considered after the 600 nm measured value was subtracted from that at 532 nm, then evaluated with an extinction coefficient of 155 mM^{-1} cm^{-1}.

Total phenolic content

Folin-Ciocalteu reagent (Singleton and Rossi 1965) was used to determine the total phenolic content (TPC). A 100 μL leaves extract (70% methanol) and 750 μL of 2% sodium carbonate were mixed and reacted with diluted Folin-Ciocalteu reagent (750 μL) for 45 min in the dark (around 20°C). The absorbance at 765 nm was measured against deionized water as blank. TPC values were calculated using a gallic acid-prepared standard curve. The results are given in mg of gallic acid equivalents per mg of fresh weight (mg GAE/ mg FW).

Total flavonoid content

Ahmed *et al*. (2021) method was used to extract total flavonoids content (TFC). Leaf samples (100 mg) lysed in 5 mL methanol (80%). The homogenates incubated at 200×*g*, 2 h and room temperature before being centrifuged at 8000×*g* for 5 min. The pellet was extracted a second time. The supernatants were then combined for determination. Aluminum chloride method of Pękal and Pyrzynska (2014) was used to determine TFC. A 100 μL of crude extract was mixed with 100 μL of 2% aluminum chloride, 20 μL of glacial acetic acid and 200 L of 100% methanol. The reaction mixture incubated for 30 min at room temperature. The absorbance was measured at 425 nm. Quercetin was used as standard at concentrations of 0.5, 1, 2, 5, 10 and 20 μg mL^{-1}.

Statistical analysis

Three independent biological experiments were performed. The findings were presented as mean± SD of 4 replicates. At a 95% confidence level ($P \leq 0.05$), one-way ANOVA was used, followed by Tukey's honest significance test, to compare the means of statistically different parameters. The data was statistically analyzed using GraphPad Prism 8.0. (GraphPad Software Inc., San Diego, CA, USA).

Results

Plant growth parameters

The growth parameters of *C. annuum* plants exposed to 150 m*M* salt stress were examined. The shoot length did not show any significant change compared to the control (Fig. 2), indicating no adverse effect of salt stress on the shoot growth. However, the root length was significantly reduced by 0.75-fold compared to the control after 9 days and by 1.2-fold compared to the control after 6 days, indicating a negative effect of salt stress on root growth (Fig. 3).

Free proline accumulation

The results showed that free proline content in *C. annuum* leaves increased significantly after exposure to salt stress (Fig. 4). After 6 and 9 days of salt treatment, the free proline content increased by 3.8- and 6.5-fold, respectively, compared to the control circumstances.

Photosynthetic pigments contents

Chlorophyll *a* content decreased significantly by 0.7-fold after 6 days of salinity exposure, but this decrease did not persist after 9 days of treatment when compared to non-stressed plants. However, chlorophyll *b* contents also decreased significantly by 0.6- and 0.8-fold after 6 and 9 days of salinity exposure, respectively, compared to the control (Fig. 5). The total chlorophyll content was reduced by 30%

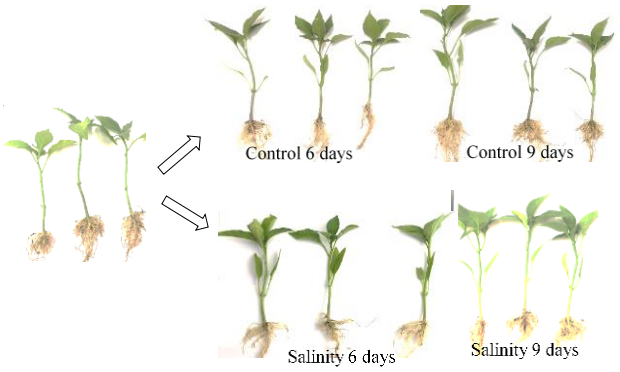

Fig. 1: Growth condition scheme to study the effect of salinity stress

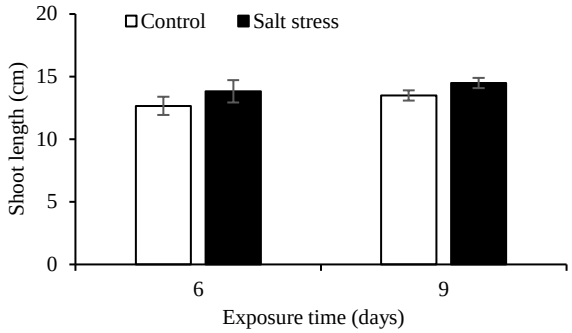

Fig. 2: Effects of salinity stress on the shoot length of *C. annuum*. The impact of various salinity stress time points in comparison to the control shoot length. Data showing replicate means ± SD. Significant differences are indicated by different letters (Tukeys test; $P \leq 0.05$)

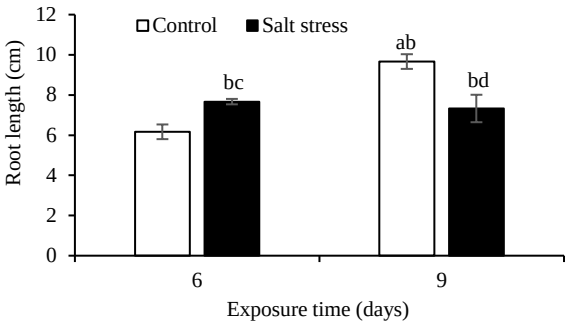

Fig. 3: Effects of salinity stress on the root length of *C. annuum*. The impact of various salinity stress time points in comparison to the control root length. Data showing replicate means ± SD. Significant differences are indicated by different letters (Tukeys test; $P \leq 0.05$)

after the duration of salinity exposure. However, carotenoid content increased significantly in response to 9 days of treatment, reaching 3-fold the control (Fig. 6). Additionally, anthocyanin content increased significantly by 2.3- and 2.9-fold after 6 and 9 days of salinity exposure, respectively, compared to non-stressed plants (Fig. 7).

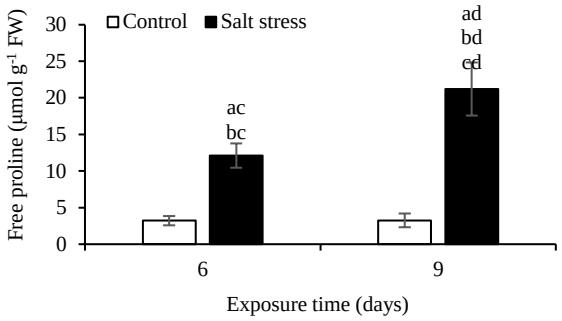

Fig. 4: Effects of salinity stress on proline contents in the leaves of *C. annuum*. The impact of various salinity stress time points in comparison to the control proline contents. Data showing replicate means ± SD. Significant differences are indicated by different letters (Tukeys test; $P \leq 0.05$)

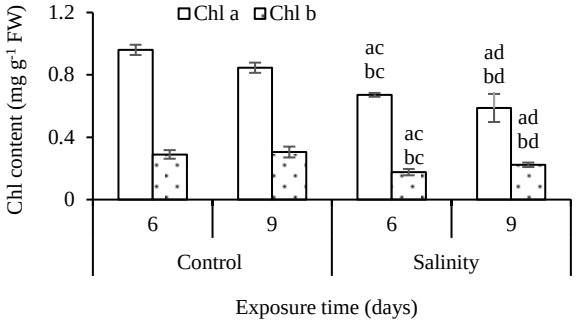

Fig. 5: Effects of salinity stress on chlorophyll *a* and *b* contents in the leaves of *C. annuum*. The impact of various salinity stress time points in comparison to the control chlorophyll *a* and *b* contents. Data showing replicate means ± SD. Significant differences are indicated by different letters (Tukeys test; $P \leq 0.05$)

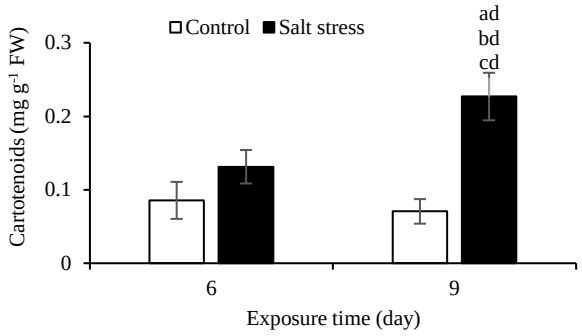

Fig. 6: Carotenoids accumulation level in the leaves of *C. annuum* subjected to salinity stress. The impact of various salinity stress time points in comparison to the control carotenoids contents. Data showing replicate means ± SD. Significant differences are indicated by different letters (Tukeys test; $P \leq 0.05$)

Oxidative stress markers

Hydrogen peroxide level: The effects of salinity stress on H_2O_2 content in *C. annuum* leaves were examined and the results are presented in Fig. 8. The H_2O_2 levels increased

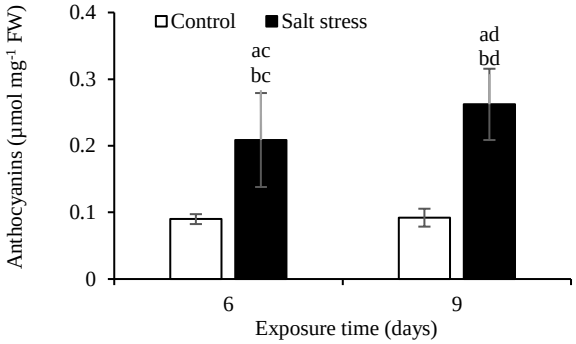

Fig. 7: Anthocyanins accumulation level in the leaves of *C. annuum* subjected to salinity stress. The impact of various salinity stress time points in comparison to the control Anthocyanins contents. Data showing replicate means ± SD. Significant differences are indicated by different letters (Tukeys test; $P \leq 0.05$)

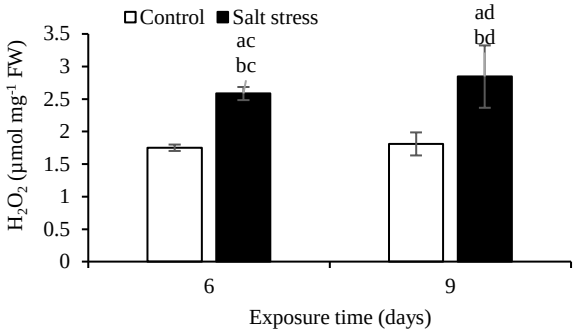

Fig. 8: Effects of salinity stress on H_2O_2 contents in the leaves of *C. annuum*. The impact of various salinity stress time points in comparison to the control H_2O_2 contents. Data showing replicate means ± SD. Significant differences are indicated by different letters (Tukeys test; $P \leq 0.05$)

significantly by 1.5-fold and 1.6-fold after 6 and 9 days of 150 m*M* NaCl treatment, respectively, compared to the control plants.

Effects on lipid peroxidation level: In this study, salinity stress induced H_2O_2 production, which can cause oxidative damage to leaves. The lipid peroxidation level was examined by measuring the MDA level in the leaves (Fig. 9). The results showed that after six and nine days of salinity exposure, the MDA levels were significantly elevated by 4.4- and 6.8-fold, respectively, compared to the control plants.

Non-enzymatic antioxidant

Total phenolic contents (TPC): The TPC level as a pattern of nonenzymatic antioxidants was evaluated in this study (Fig. 10). The results showed that after 9 days of salinity exposure, the TPC levels in the plant leaves increased by 1.2-fold compared to the control conditions.

Total flavonoid contents: This study also evaluated total flavonoid content (TFC) as a measure of non-enzymatic antioxidants, as shown in Fig. 11. The results showed that after 6 and 9 days of salinity exposure, the TFC levels in the

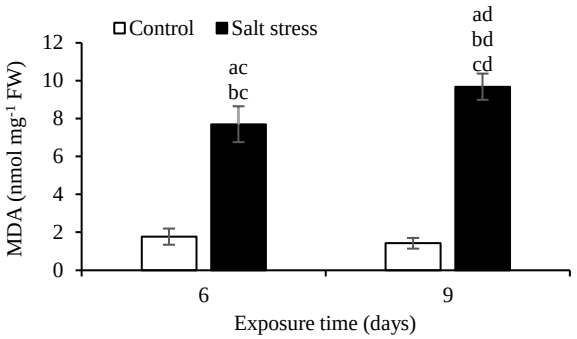

Fig. 9: Effects of salinity stress on lipid peroxidation levels (MDA levels) in the leaves of *C. annuum*. The impact of various salinity stress time points in comparison to the control lipid peroxidation levels. Data showing replicate means ± SD. Significant differences are indicated by different letters (Tukeys test; $P \leq 0.05$)

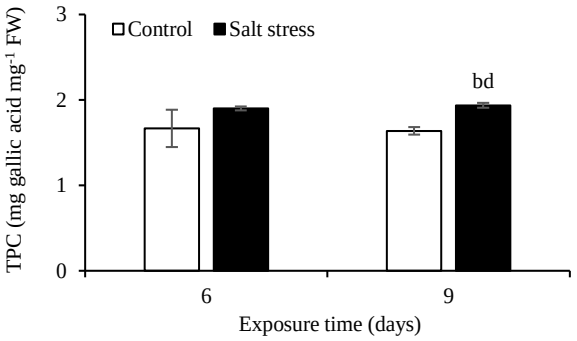

Fig. 10: Total phenolic contents (TPC) in the leaves of *C. annuum* subjected to salinity stress. The impact of various salinity stress time points in comparison to the control total phenolic contents. Data showing replicate means ± SD. Significant differences are indicated by different letters (Tukeys test; $P \leq 0.05$)

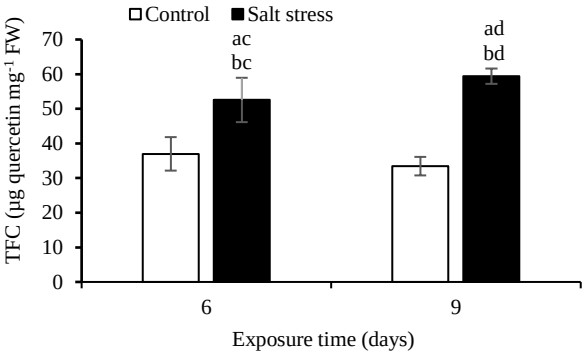

Fig. 11: Total flavonoid contents (TFC) in the leaves of *C. annuum* subjected to salinity stress. The impact of various salinity stress time points in comparison to the control total flavonoid contents. Data showing replicate means ± SD. Significant differences are indicated by different letters (Tukeys test; $P \leq 0.05$)

leaves of *C. annuum* increased by 1.4- and 1.8-fold, respectively, compared to the control conditions.

Discussion

Salinity stress has a broad adverse effect on plant performance (Ghane *et al*. 2011; Hoque *et al*. 2023). However, *Capsicum* metabolome response variation during salinity is cultivar-, fruit part-, environment and salinity level-dependent (De Pascale *et al*. 2003; Niu *et al*. 2010; Zamljen *et al*. 2022). In the current study, the application of 150 m*M* NaCl had an intense effect on *C. annuum* growth. The *C. annuum* shoot length was less affected. While the root length was significantly reduced after 6 days of stress, it improved after 9 days of treatments (Fig. 2 and 3). These findings imply that in *C. annuum* under salinity stress, plant growth is reduced due to the osmotic effect of the salt outside the roots that enhanced the plant low water potential which appeared as a non-significantly shoot length increase and significant root length reduction, in particular, early salt stress exposure. Moreover, long-term exposure may lead to ion toxicity and imbalance excreted by salinity that ultimately evoked internal injury (De Pascale *et al*. 2003; Ziaf *et al*. 2009; Bojórquez-Quintal *et al*. 2014; Butt *et al*. 2021).

To avoid the negative effects of salinity, *C. annuum* has intrinsic tolerance mechanisms. Compatible solutes such as proline provide osmotic adjustment and non-enzymatic antioxidants due to its ability to scavenge ROS (Gupta and Huang 2014). Free proline accumulation is an important protective mechanism for dealing with a variety of stresses including salinity (Kishor and Sreenivasulu 2014; Hasanuzzaman *et al.* 2021). Thus, free proline content increased significantly in *C. annuum* leaves in this study as the duration of salinity treatment extended. For proline contributing to osmotic adjustment, protecting role and ROS quenching is considered one of the salt tolerance mechanisms that as other previous findings have already described (Bojórquez-Quintal *et al*. 2014; Gupta and Huang 2014; Hand *et al*. 2017; Butt *et al*. 2021; López-Serrano *et al*. 2021). As an indicator of salt stress severity, free proline content aggrandizement in response to salinity duration exposure was beside the reduction of chlorophyll content.

Free proline accumulation in *C. annuum* leaves has been suggested to maintain chlorophyll content and turgor in order to protect photosynthetic activity under salt stress conditions (Hnilickova *et al*. 2021). This is because in this study, the total chlorophyll content was not reduced by more than 30%, and the chlorophyll *a* and chlorophyll *b* content were maintained at 0.7 and 0.8-fold, respectively, despite the fact that the salinity duration exposure was extended (Fig. 5). A decrease in total chlorophyll content may be observed due to ion accumulation, which causes ion toxicity, preventing pigment formation due to the destruction of their structure with ROS and inhibiting biosynthesis of new chlorophylls (Supanjani and Lee 2006; Ziaf *et al*. 2009; López-Serrano *et al*. 2021). Abiotic stresses trigger the overproduction of ROS, which is scavenged by non-enzymatic antioxidants such as phenolics, α-tocopherol, carotenoids, flavonoids, ascorbate, glutathione, and even proline (Sachdev *et al*. 2021).

The accumulation of H_2O_2 in *C. annuum* leaves has been identified as a signal of ROS damage (López-Serrano *et al*. 2021). For that, the severity of salt stress of this work was experienced by *C. annuum* leaves that accumulate H_2O_2. This increase in H_2O_2 content may be due to the fact that ROS have vital roles under stress conditions such as stress signaling molecules or they lead to oxidative stress during various stresses (Alsharafa 2017; Alkhsabah *et al*. 2018; Al-Sammarraie *et al*. 2020). MDA, or lipid peroxidation, is a major determinant of oxidative damage caused by salt stress (Hnilickova *et al*. 2021). *C. annuum* exhibited higher leaves MDA contents with reduced total chlorophyll contents in this study (Fig. 5 and 9), indicating that *C. annuum* is moderately sensitive to salt stress as similarly reported studies exhibit the negative effects of salinity have been attributed to the disruption in physiological and biochemical events of *C. annuum* (Bojórquez-Quintal *et al*. 2014; Suarez *et al*. 2021). In previous studies, positive connections between salinity stress and carotenoids contents as antioxidants were observed in the leaves of *C. annuum* (Ziaf *et al*. 2009; Gammoudi *et al*. 2016). Hence, carotenoids accumulation is one of the salt tolerance mechanisms in the leaves of *C. annuum* that appear to be salt severity and duration of exposure dependent mechanism. Due to the salt concentration, water availability and uptake being restricted, resulting in a decrease in plant water content, ion toxicity, and ROS elevation, which alters the metabolic processes inside plant cells. For that, the current finding of carotenoids accumulation reveals the duration of salt stress exposure as a determinant factor (Ziaf *et al*. 2009; Hnilickova *et al*. 2021).

To achieve salt tolerance, many biochemical and physiological processes are activated. Anthocyanins consider stress signals, ROS scavenger and photoprotectants (Šamec *et al*. 2021). Salinity induces the accumulation of anthocyanins in *C. annuum* (Abbas *et al*. 2013; Genzel *et al*. 2021). In this study, the enhanced anthocyanins contents in *C. annuum* seemed to contribute to the higher salt tolerance achieved through the prevention of stress-induced oxidative damage (Abbas *et al*. 2013; Genzel *et al*. 2021). Flavonoid and phenolic contents among various metabolome indices allow the estimation of stress intensity. Hand *et al*. (2017) differentiated the role of osmolytes accumulation and antioxidant compounds of *C. annuum* in response to the effects of salinity. The effects of salinity intensity on flavonoid and phenolic content in *C. annuum* increased the levels of two leaves antioxidants appear to contribute to salinity tolerance in *C. annuum* (Genzel *et al*. 2021).

Conclusion

The main flexible salt tolerance mechanisms of *C. annuum* were osmolyte accumulation (proline) and antioxidants (carotenoids, anthocyanins, total phenolic compounds, and total flavonoids), among other molecular and biochemical mechanisms, which were systematic to mitigate osmatic stress, ion toxicity, and oxidative damage. Further investigations on phytohormones and polyamines may be studied under salt stress conditions.

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Author Contributions

KA administers the project, performed the research, and wrote the article.

Conflict of Interest

The author does not have any conflict of interest to declare.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper.

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