


Choline Chloride Mediates Salinity Tolerance in Cluster Bean (*Cyamopsis tetragonoloba* L.) by Improving Growth, Oxidative Defense, and Secondary Metabolism

Dose-Response:
An International Journal
October-December 2021:1–19
© The Author(s) 2021
Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/15593258211055026
journals.sagepub.com/home/dos


Saima Riaz, PhD¹, Iqbal Hussain, PhD¹ , Muhammad Ibrahim, PhD², Rizwan Rasheed, PhD¹, and Muhammad Arslan Ashraf, PhD¹

Abstract

Choline chloride (CC) application enhanced the tolerance of cluster bean (*Cyamopsis tetragonoloba* L.) against salinity stress. The aim of the study was to determine the protective role of CC on plant growth, photosynthesis, and biochemical indicators of oxidative stress. The seeds of BR-99 (tolerant) and BR-2017 (sensitive) were surface sterilized and sown in plastic pots containing river sandy soil. The design of the experiments was completely randomized with 4 replicates per treatment. Three weeks after germination, salinity (150 mM) was imposed. Then plants were sprayed with different concentrations of CC (3, 5, and 10 mM), while normal plants were sprayed with distilled water. Salinity decreased growth attributes, relative water contents, photosynthetic attributes, total soluble proteins, total free amino acids, phenolic, flavonoids, ascorbic acid, proline, and glycine betaine and increased the levels of oxidative stress indicators. However, the application of CC (particularly 5 mM) improved growth attributes, photosynthetic pigments, and activities of antioxidant compounds by reducing the levels of H₂O₂, malondialdehyde in salt-stressed plants in both cluster bean varieties. BR-99 variety showed more tolerance to salinity stress than that of BR-2017 in the form of greater oxidative defense and osmotic adjustment and clear from greater plant dry masses. Thus, our results showed that the application of CC (5 mM) is an efficient strategy for field use in the areas, where salt stress soils limit agriculture production.

Keywords

choline chloride, oxidative stress, antioxidant compounds, salinity, cluster bean, glycine betaine

Introduction



Abiotic stress is envisaged as a major threat to the world agricultural system. Salinity is a severe problem in the

world's arid and semi-arid regions, notably impeding crop value and production, often occurring together with alkaline stress.^{1,2} Besides, salinity stress individually or in combination is a significant environmental factor that hampers the normal plant growth production capacity.³ According to estimates, the salinity of soil affects 6-10% of the total

¹Department of Botany, Government College University, Faisalabad-Pakistan
²Department of Applied Chemistry, Government College University, Faisalabad, Pakistan

Corresponding Author:

Iqbal Hussain, Department of Botany, Government College University, Faisalabad-38000, Pakistan.
Email: driqbal@gcuf.edu.pk



Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (<https://creativecommons.org/licenses/by-nc/4.0/>) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (<https://us.sagepub.com/en-us/nam/open-access-at-sage>).

world's area approximately, over 800 million hectares of land are harmed by salinity.⁴⁻⁶ According to another estimate, the saline-alkaline problem affects 10% of the world's total fertile land area, causing a considerable number of irrigated lands to fallow.^{7,8} Some studies show that about 77% of major saline land is found in Asia's arid and semi-arid areas particularly Pakistan, where about 10 million hectares (12.9%) of the total area is categorized as saline.⁹⁻¹¹ The salinity inhibits the growth at various development stages from germination to reproductive stages,¹²⁻¹⁴ results up to 70% economic crop loss depending upon types of plant.¹⁵⁻¹⁷ Thus reduction in their total yield is making the agriculture sector handicapped.¹⁸

Saline salts inhibit the growth, photosynthetic potential, and ion balance in maize,¹⁹ canola,²⁰ fenugreek,²¹ and sunflower.²² Numerous research studies have recognized the underlying plant defensive responses to salt, leading to the formation of agricultural plants with improved salinity tolerance.^{23,24} The soil salinity disturbs the root's functioning for minerals transport from soil atmosphere to plant transpiration stream with excessive damage,²⁵ and disturbs ionic homeostasis.²⁶ Furthermore, this will result in the increased photorespiration and decreased photosynthesis, impaired chlorophyll synthesis,^{27,28} and loss of thylakoid membrane's integrity.²⁹ Plants under salinity stress produce a significant amount of reactive oxygen generation (ROS) that induces progressive lipid peroxidation³⁰ and alteration in enzymatic antioxidant activities.³¹ Oxidative stress due to the peroxidation of lipids evokes stress responses in plants, triggering enzymatic and non-enzymatic antioxidants to cope with the oxidative injury.³² In response to environmental stresses, plants reprogram some defense responses. The accumulation of osmoregulatory compounds and selective ions absorption are necessary for defensive responses in plants against ions' excess toxicity and osmotic stress.³³ Plants under salinity stress often encounter osmotic stress and ion toxicity. Though plants have various defense mechanisms to acclimate these conditions,³⁴ and accumulation of osmoprotectants such as proline, soluble sugars, and glycine betaine (GB) to maintain steady water uptake in plants.^{35,36}

Choline is a water-soluble vitamin³⁷ recognized as an osmoprotectant for its defensive role against multiple stress.^{38,39} It is an essential component of membrane phospholipids^{40,41} that ensures the membrane stability, fluidity, and integrity of Mn-cluster and peripheral proteins of photosystems, particularly in cyanobacteria and many plant species^{42,43} under stress conditions.⁴⁴ Furthermore, choline is a vital metabolite for plant growth and development.^{45,46} Exogenous application of choline in the form of choline chloride (CC) is a novel approach to enhance salt tolerance in plants, cyanobacteria, and their nutritional value^{39,46}. It is also used to accelerate the growth of animal-like chicken and in vitro improved the growth of grasses and morphogenesis of many other plant species.⁴⁷⁻⁴⁹

Cluster bean (*Cyamopsis tetragonoloba* L.) is a multi-purpose leguminous crop, widely grown in the arid and semi-arid regions of India (80%), Pakistan (15%), Sudan, USA, and SA (5%).⁵⁰⁻⁵² In Pakistan, around 15% of total worldwide cluster bean production generally, and more than .181 million hectares area for cluster bean production is cultivated mostly in Punjab, and Sindh province⁵³⁻⁵⁵. It has wide application from the food industry to textile, medicine, dyes or varnish, cosmetics, explosives, and petroleum industry.⁵⁶⁻⁵⁸ The crop is being exploited for its active phytochemical compound in the cure of cancer, ulcer, diabetic, anti-asthmatic, and anti-inflammatory properties.⁵⁹⁻⁶¹ Therefore in the future, there is a need to manage the previously mentioned problem and its impacts in the production of this important crop. Our main objective was to assess the potential of CC to regulate the ROS metabolism and osmotic adjustment in cluster bean under saline conditions and to quantify the proper dose of CC that can effectively mitigate the toxic effect of salt stress on young cluster bean plants.

Methods

Plant Material and Growth Conditions

The present study was conducted at the research site of the Botany Department, Government College University Faisalabad, Pakistan, during the summer season (July 2018). Seeds of 2 cluster bean varieties having the differential salt tolerance capacity namely BR-99 tolerant⁶² and BR-2017 intolerant⁶³ were obtained from Arid Zone Research Institute Bhakkar, Pakistan. During the experiment, the maximum and minimum temperature $38.5 \pm 2^\circ\text{C}$ and $26 \pm 2^\circ\text{C}$ with an average 69.0% to 80.1% relative humidity, and photosynthetically active radiation (PAR; $1262 \pm 339 \mu\text{mol (photon) m}^{-2} \text{s}^{-1}$) was observed. Sodium chloride (NaCl) was used as the source for salt stress. Seeds were surface sterilized with .5% sodium hypochlorite for 5 minutes before use. Afterward, 10 seeds were sown in each plastic pot (22 cm height \times 30 cm diameter) containing 10 kg pure dry river sand. Prior to use, river sand was thoroughly washed with distilled water and then oven-dried. After germination, plants were thinned to 4 plants per pot; each pot representing 1 replicate. Plants were fertilized on alternative days in half-strength Hoagland's nutrient solution⁶⁴ to retain moisture contents to avoid drought stress. After 20 days after germination, plants of cluster bean were subjected to salinity stress (150 mM). For this purpose, solution-containing salt (50 mM) was prepared in half-strength Hoagland's nutrient solution. An increment of 50 mM salt solution was used to avoid any osmotic shock to the plants each day until the desired salinity level (150 mM) was attained. Choline chloride was dissolved in distilled water containing few drops of dimethyl sulfoxide. Saline conditions were maintained for 3 weeks. Foliar spray of CC (Merck, pH 5.5) solution having different levels (3, 5, and 10 mM)⁶⁵ with .1% tween-20 as a surfactant was applied at 8-9 am for

2 weeks after the imposition of salinity stress. After 3 days of recovery, the plants were sprayed until thoroughly wet for two weeks. Aluminum foil was wrapped around the base of the plant to prevent any possible mix-up of CC solution in the soil. The average volume of CC applied per plant was 200 mL. For comparison, plants were also sprayed with distilled water. 2 weeks after foliar application of CC, 3 plants were harvested from each pot for recording the growth attributes. The penultimate leaf of the fourth plant was harvested to measure the physiological and biochemical attributes. The experiment was conducted in a completely randomized design (CRD) with 4 replications for each treatment.

Growth Attributes

Plants were harvested, thoroughly rinsed in deionized water, and blotted dry with a paper or towel before recording root and shoot fresh weight (SFW). The dry weight of those samples was estimated that reached a constant weight after oven drying at 80°C for 1 week. The leaf area of plants was measured from the method of grid counting. Leaves were placed on a millimeter graph paper (1 cm grid paper), and the leaf outline was drawn on a millimeter graph paper, and then the occupied block within the outlined area was counted. Plant leaf area was measured by using the following equation⁶⁶

$$\text{Leaf area (cm}^2\text{)} = N \times B$$

where N is the number of blocks (1 cm) covered by leaf and B is one block area in the graph paper.

Photosynthetic Pigments

Chlorophyll (a, b) and carotenoids were measured from the fresh leaf with the following method of Lichtenthaler.⁶⁷ Fresh leaf (.15 g) was homogenized in 10 mL aqueous acetone (80%). The mixture was centrifuged at 4000 r/min for 10 minutes. The absorbance of the supernatant was read at 663, 645, and 480 nm on a UV-Visible (Hitachi U –2910, Tokyo, JP) spectrophotometer. The calculation of chlorophyll (a, b) molecules was performed with the help of the following formula

$$\text{Chl.a (mg/g FW)} = \frac{12.7 (\text{OD663} - 2.69(\text{OD645})) \times V}{1000 \times W}$$

$$\text{Chl.b (mg/g FW)} = \frac{12.9 (\text{OD645} - 4.68(\text{OD663})) \times V}{1000 \times W}$$

$$\text{Total Chl. (mg/g FW)} = \frac{20.2 (\text{OD645} - 8.02(\text{OD663})) \times V}{1000 \times W}$$

Carotenoids (mg/g FW)

$$= \{\text{OD480} + (0.114 \times \text{OD663}) - (0.638 \times \text{OD645})\}$$

Leaf Relative Water Content

Leaf discs (1.0 cm in diameter) of penultimate leaves were sampled with a cork borer. The fresh weight of ten leaf discs was calculated. Turgid weight was determined by immersing them in distilled water overnight at 4°C in darkness. Then leaf discs were dried in an oven for 48 hours at 70°C for measuring dry weight. Relative water content was assessed following the equation proposed by Cornic.⁶⁸

$$\text{LRWC} = \left[\left(\frac{\text{Fresh weight} - \text{dry weight}}{\text{Turgid weight} - \text{dry weight}} \right) \right] \times 100$$

Hydrogen Peroxide Content

Fresh leaf tissue (.15 g) was crushed in 2.5 mL of .1% trichloroacetic acid (TCA) to extract H₂O₂. The resultant homogenate was centrifuged at 12 000g for 15 minutes. The supernatant (.5 mL) was reacted with KI (1.0 M) and .5 mL potassium phosphate buffer (50 mM; pH 7.5). The absorbance of the mixture was read at 390 nm wavelength using a UV-Visible spectrophotometer.⁶⁹

Malondialdehyde Content

Malondialdehyde content was measured according to the procedure of De Vos et al.⁷⁰ Fresh leaf (.15 g) was crushed in 1.5 mL TCA solution (10%). After that, the samples were centrifuged at 15 000g for 15 minutes to collect the supernatant. The supernatant (2 mL) was mixed with .6% thiobarbituric acid (TBA-2 mL). The reaction mixtures were heated at 100°C in a water bath. The samples were removed from the water bath and allowed to cool down to room temperature before taking absorbance at the suggested wavelength (600 and 532 nm).

Proline Contents

Fresh leaf tissue (.25 g) was homogenized in 5 mL sulfosalicylic acid (3%). The homogenate was centrifuged at 10 000g for 5 minutes to obtain the supernatant. The supernatant was used for estimating proline content. The reaction mixture consists of 2 mL of the supernatant, 2 mL of acid-ninhydrin (2.5%), and 2 mL of glacial acetic acid. The reaction mixture was boiled at 100°C for 25 minutes. Then the mixture was cooled at room temperature and extracted with 4 mL of toluene. The supernatant was used to read the absorbance at 520 nm with a spectrophotometer.⁷¹ Proline was calculated

using a standard curve and expressed in the form of $\mu\text{mol g}^{-1}$ fresh weight.

Ascorbic Acid Contents

Fresh leaf (.15 g) was homogenized in 5% TCA solution and filtered. 2 mL filtrate was reacted with 2% 2, 4 dinitrophenyl hydrazine (1.0 mL) and 1 drop of thiourea (10%) dissolved in ethanol. The reaction mixture was incubated in the water bath for 20 minutes at 98°C. After that, the samples were removed from the water bath and allowed to cool down to 25°C followed by the addition of 80% H_2SO_4 (2.5 mL) at 4°C. The absorbance of the reaction mixture was read at 530 nm using a spectrophotometer.⁷²

Total Flavonoids Contents

Flavonoids were determined according to the procedure of Zhishen et al.⁷³ Fresh leaf (.15 g) was ground in aqueous ethanol (80%) and the resulting mixture was centrifuged to obtain the supernatant. 1 mL of extract was reacted with AlCl_3 and NaNO_2 (300 μL each). After that 2 mL NaOH (1 M) was added to the mixture followed by the 5 minutes incubation at room temperature. The volume of the mixture was made to 10 mL with distilled water. The absorbance of the samples was taken at 510 nm using a spectrophotometer.

Total Phenolic Contents

Julkunen-Tiitto⁷⁴ method was used to estimate the total phenolic contents from fresh leaf. Aqueous ethanol (80%) was used to homogenate the fresh leaf sample (.15 g) and centrifuged. Then 100 μL supernatant was reacted with 2 mL folin-ciocalteu phenol reagent and 5 mL of 20% Na_2CO_3 . The volume of the reaction solution was made to 10 mL using distilled water. The absorbance of the mixture was measured at the advised wavelength (750 nm).

Total Free Amino Acids

Ninhydrin assay described by Hamilton and Van Slyke⁷⁵ was used to estimate the total free amino acid (TFAA) contents. Fresh leaf material (.15 g) homogenized in 5 mL of 80% ethanol. The homogenate was centrifuged at 15 000g for 20 minutes. .1 mL supernatant reacted with 1 mL of ninhydrin reagents (2% ninhydrin) and 1 mL pyridine (10%). The reaction mixture was shaken vigorously and then incubated in the water bath at 92°C for 30 minutes. After cooling, the absorbance was noted at 570 nm using a spectrophotometer. Ethanol (80%) was used as a blank. Total free amino acids were calculated using a standard curve and expressed in the form of mg g^{-1} fresh weight.

Total Soluble Protein

Fresh leaf tissues (.15 g) were homogenized in 10 mL chilled phosphate buffer (50 mM; pH 7.5). The supernatant was obtained after centrifugation at 12 000g for 20 minutes at 4°C. The supernatant (enzyme extract) was mixed with Bradford reagent and absorbance was taken at 595 nm for protein estimation.⁷⁶

Anthocyanin Content

Hodges and Nozzolillo⁷⁷ method was used for the estimation of total anthocyanin contents. Fresh leaves (.15 g) were grinded in 2 mL of acidified methanol with 1% HCl then the material was centrifuged at 12 000g. The resulting supernatant was used to measure the absorbance at 530 and 657 nm. Anthocyanin content was calculated following the equation proposed by Mita et al.⁷⁸

$$\text{Anthocyanin (mg/g FW)} = (\text{OD}_{530} - 0.25 \times \text{OD}_{657})$$

Total Soluble Sugar. Total soluble sugar (TSS) was measured with the procedure of Yemm and Willis.⁷⁹ Fresh leaf material (.15 g) extracted in 80% aqueous ethanol. 3 mL anthrone reagent (prepared in 75% sulfuric acid) mixed with 100 μL of the extract. The reaction mixture was carried out in a water bath at 95°C for 10 minutes. After incubation, test tubes were cooled down at 25°C, and absorbance was measured at 625 nm. Total soluble sugars were calculated with the help of a standard curve made from the solution of known concentration and expressed in the form of mg g^{-1} fresh weight.

Reducing Sugar

Reducing sugar (RS) was estimated with the procedure of Nelson.⁸⁰ Fresh leaf material (.15 g) extracted in 80% aqueous ethanol. 5 mL of O-toluidine (6%) mixed with 1 mL of aqueous ethanol extract. Then reaction mixture was heated in a water bath at 95°C for 20 minutes. After cooling the reaction mixture, the absorbance of RS was measured at 630 nm. Reducing sugars were calculated with the help of a standard curve. The data of RSs was expressed as mg g^{-1} fresh weight.

Antioxidant Enzymes Assay

Fresh leaf (.5 g) was powdered in liquid nitrogen, and homogenized in 50 mM potassium phosphate buffer (50 mM; pH 7.5). The supernatant was obtained after centrifugation at 12 000g for 20 minutes at 4°C. The supernatant (enzyme extract) was used for the measurement of enzyme activities. The reaction solution for catalase (CAT) activity contained 1.9 mL potassium phosphate buffer (50 mM; pH 7.0), 1 mL H_2O_2 (5.9 mM), and .1 mL enzyme extract. The reaction mixture was illuminated and the change in absorbance was

read at 240 nm with a spectrophotometer.⁸¹ The peroxidase (POD) activity was also estimated using the method of Chance and Maehly.⁸¹ The enzyme extract (100 μ L) was mixed with H₂O₂ (40 mM) and guaiacol (20 mM). The guaiacol oxidation rate in H₂O₂ presence read at 470 nm indicates the enzyme activity. Superoxide dismutase (SOD) activity was measured by the method of Gong et al.⁸² The reaction solution (3 mL) contained 100 μ L enzyme extract, 50 mM potassium phosphate buffer, 56 mM NBT (nitroblue tetrazolium), 1.17 mM riboflavin, and 10 mM methionine. The reaction mixture was illuminated under a light bulb for 10 minutes. The absorbance was recorded at 560 nm. The specific activity of each enzyme was expressed as units of enzyme/mg protein.

Statistical Analysis

The experiment was performed in a CRD in a factorial arrangement. Each treatment had 4 replications. The STATISTICS software (version 8.1) was used to calculate the analysis of variance of the data. The least significant difference was used to compare means at $P \leq .05$. The different correlations in the data were drawn using RStudio.

Results

Growth Attributes

Under salt stress conditions (150 mM NaCl), shoot and root lengths (RLs) of cluster bean are significantly ($P \leq .001$) reduced as compared to controls. Plants of BR-99 and BR-2017 varieties subjected to 150 mM NaCl showed a reduction in the shoot (23% and 29%) and root lengths (33% and 47%). Choline chloride (0, 3, 5, and 10 mM) significantly ($P \leq .001$) reduced the harmful effects of salt stress on these development characteristics. Plants given 5 mM CC exhibited greater shoot (33% and 28%) and root lengths (91% and 109%) under salt and control conditions in both BR-99 and BR-2017 varieties, respectively. While 10 mM CC decreased the shoot length (14% and 20%) under both conditions, and decreased (18%) the root length in variety BR-99 under salinity and increase (18%) under control conditions, respectively. Further, 10 mM CC increased (25% and 50%) the root length in BR-2017 under both control and stress conditions, respectively (Table 2). Shoot (31% and 38%) and root fresh (21% and 25%) and shoot (24% and 26%) and root dry biomass (19% and 21%) reduced remarkably ($P \leq .001$) in plants of BR-99 and BR-2017 under salt stress, respectively. Plants sprayed with water increased the shoot (29% and 37%) and root fresh weight (RFW) (25% and 23%) in both varieties, respectively. Exogenous CC (3, 5, and 10 mM) significantly ($P \leq .001$) improved shoot–root fresh and dry biomass, especially in plants given 5 mM CC under salt stress conditions (Table 2). Plants sprayed with CC (5 mM) showed greater shoot (29%, 17% and 50%, 37%) and root fresh biomass (17%, 44% and 23%, 81%) under salt and control conditions in both BR-99 and BR-2017

varieties, respectively. We noticed a significant ($P \leq .001$) abridge in the root (19% and 21%) and shoot (24% and 26%) dry weights in plants exposed to 150 mM salt stress. Water spray induced the most significant increase in their roots (34% and 26%) and shoots (27% and 28%) dry mass attributes in both BR-99 and BR-2017, respectively. The salt stress effects on root and shoot dry weight (SDW) were considerably ($P \leq .001$) reduced by CC (3 mM and 5 mM), with the maximum improvement shown in plants of BR-99 (85%, 66% and 43%, 52%) and BR-2017 (80%, 68% and 63%, 96%) fed 5 mM CC under both conditions, respectively. Although exogenous CC (10 mM) reduced the shoot and root dry biomass in both varieties under both conditions, greatly reduced in BR-2017 was recorded (Table 2). Salt stress significantly ($P \leq .001$) declined (26% and 33%) the leaf area in both varieties of cluster bean plants. Water spray caused a substantial increase in this variable in both BR-99 (38%) and BR-2017 (44%) under stress conditions. Plants administered CC (3 and 5 mM) had significantly ($P \leq .001$) increased the leaf area in BR-99 and BR-2017 under both conditions. Plants sprayed with CC (5 mM) showed leaf area under control as well as stress in both BR-99 (68% and 66%) and BR-2017 (60% and 68%), respectively. Higher CC level (10 mM) caused a substantial reduction in this variable in both varieties. In this context, leaf area was more declined in BR-2017 than BR-99 under stress conditions (Table 2).

Photosynthetic Pigments and Relative Water Contents

Salt stress significantly decreased ($P \leq .001$) photosynthetic pigments (for instance Chl. a Chl. b, total Chl, and carotenoids) in both varieties of cluster bean plants (Table 1). We have recorded a remarkable decline in Chl.a (20% and 31%), Chl.b (25% and 31%), and total Chl (23% and 31%) under salinity stress (150 mM NaCl) in both BR-99 and BR-2017, respectively. Water spray increased the chlorophyll molecules, that is, Chl.a (10%, 3% and 38%, 56%), Chl.b (–.34%, 3% and 21%, 43%), and total Chl (5%, 3% and 31%, 51%) in both BR-99 and BR-2017 under control and salt stress conditions, respectively. Plants administered CC (3 and 5 mM) had improved the chlorophyll molecules in control and stressed plants of both varieties. Under salt stress, exogenous CC (5 mM) resulted in a significant increase in chlorophyll molecules. For instance, Chl. a (48%, 44% and 66%, 83%), Chl. b (23%, 26% and 62%, 96%), and total Chl (36%, 36% and 64%, 88%) under both conditions in BR-99 and BR-2017, respectively. Plants sprayed with CC (5 mM) showed LA under control as well as stress in both BR-99 (68% and 66%) and BR-2017 (60% and 68%), respectively. A higher level of CC (10 mM) caused a substantial reduction in Chl. b contents in BR-99 (35%) and BR-2017 (22% and 28%) under both control and stress conditions, respectively. Furthermore, total Chl declined (8% and 13%) in BR-99 under both control and stress conditions (Table 3). Salinity stress (150 mM NaCl) significantly ($P \leq .001$) reduced carotenoid content in both

BR-99 (22%) and BR-2017 (33%). Water spray caused a substantial increase in this variable in BR-99 (18% and 4%) and BR-2017 (19% and 25%), respectively. Plants sprayed with CC (3, 5, and 10 mM) significantly ($P \leq .001$) reduced the harmful effects of salt stress on carotenoid contents. When plants fed 5 mM CC, this variable substantially enhanced in both BR-99 (44% and 25%) and BR-2017 (66% and 104%) under normal and stress conditions (Table 3).

Relative water contents decreased significantly ($P \leq .001$) in cluster bean under salt stress conditions (0, and 150 mM NaCl). The decrease in relative water content (RWC) was greatest in BR-2017 (50%) as compared to BR-99 (37%) under salinity (150 mM). Water spray improved the water contents in the plants of both BR-99 (14% and 24%) and BR-2017 (35% and 63%) under both conditions, respectively. Plants given CC had significantly ($P \leq .001$) enhanced the RWC in both varieties under control as well as stress conditions. Among CC levels, 5 mM CC spray greater increased the RWC (40% and 59%) in BR-99 and (72% and 151%) in BR-2017 under control and stress conditions, respectively. Although, 10 mM CC reduced this variable in both cluster bean varieties (Table 3).

Oxidative Stress Indicators

Plants exposed to salt stress significantly increased ($P \leq .001$) the MDA contents (Table 1). Plants exposed to 150 mM NaCl showed significantly higher MDA levels in both BR-99 (176%) and BR-2017 (190%) than control plants. Water spray decreased the MDA levels in both BR-99 (2% and 18%) and BR-2017 (23% and 11%) under normal and stress conditions, respectively. Under the saline condition, plants given CC (3 and 5 mM) showed a significant ($P \leq .001$) reduction in MDA levels in both varieties (Table 1). In addition, the maximum reduction in MDA levels was evident in plants of both BR-99 (38% and 62%) and BR-2017 (65% and 54%) with 5 mM CC administration under normal and stress conditions, respectively. Plant-given CC (10 mM) increased the minimal levels of MDA in both varieties, greatly in BR-2017 under stress conditions (Figure 1).

Hydrogen peroxide (H_2O_2) levels increased significantly ($P \leq .001$) in cluster bean plants under salt stress (Table 1). H_2O_2 values were higher in BR-2017 (121%) than BR-99 plants (98%) when exposed to 150 mM NaCl. Moreover,

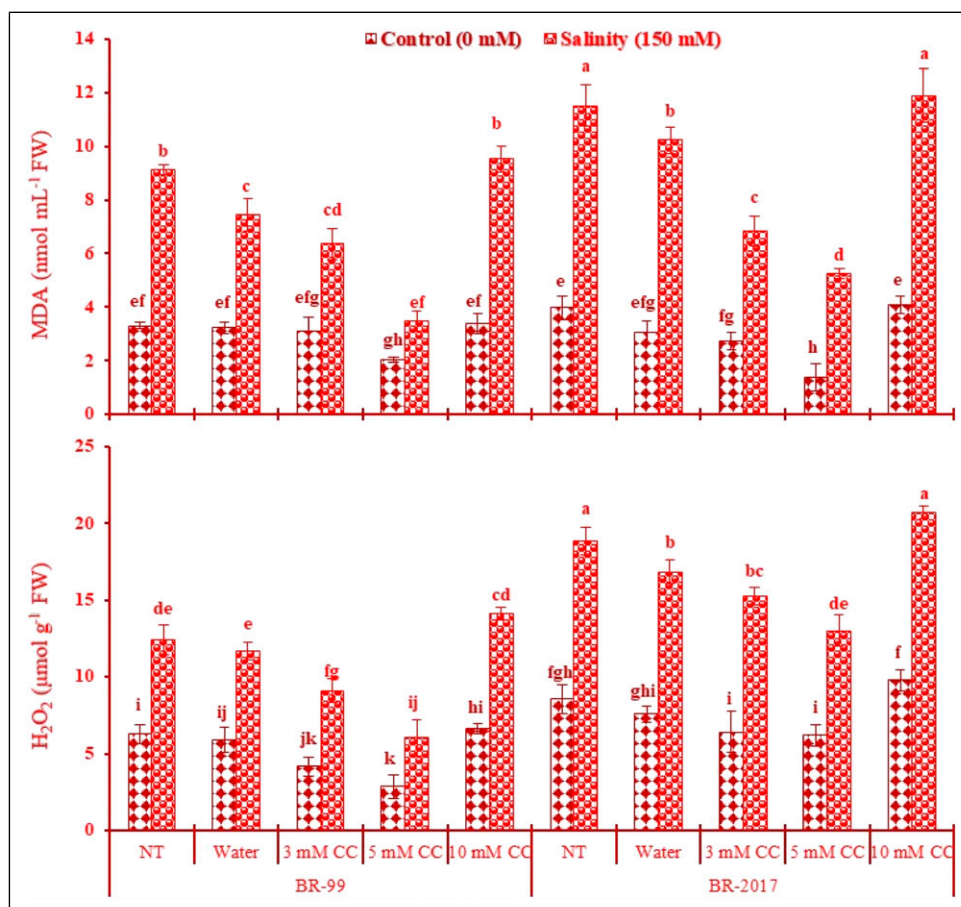


Figure 1. Changes in malondialdehyde (MDA), and hydrogen peroxide (H_2O_2) in 2 cluster beans varieties sprayed with different regimes of choline chloride (CC) under salt stress ($n = 4 \pm SE$). Bars expressed with different letters are significantly different according to using least significant difference (LSD) at $P \leq .05$.

water and CC (3 and 5 mM) treatments resulted in a significant ($P \leq .001$) reduction in this variable under salt stress in both varieties (Table 1). Likewise, minimal H_2O_2 levels were evident in both BR-99 (55% and 51%) and BR-2017 (28% and 31%) varieties with 5 mM CC administration under normal and salt stress conditions, respectively. By contrast, plants given 10 mM CC raised the H_2O_2 levels in both varieties, more evident in the BR-2017 variety (Figure 1).

Cytosolutes. Total soluble sugars (TSSs) and reducing sugars (RSs) decreased significantly ($P \leq .001$) in cluster bean plants under salt stress (Table 1). Plants exposed to 150 mM NaCl showed low levels of TSSs. Under salt stress circumstances, water spray and CC (3, 5) improved endogenous sugar levels considerably under stress conditions. Plants given 5 mM CC

showed the highest levels of soluble sugar in both BR-99 (52%) and BR-2017 (160%) under stress, respectively. In addition, exogenous application of CC (10 mM) increased the minimal levels of TSS in BR-2017 variety under salt stress (Table 4). Similarly, salt stress decreased the RS in both BR-99 (17%) and BR-2017 (22%) varieties. In stressed cluster beans plants of both varieties, CC (3 and 5 mM) substantially enhanced ($P \leq .001$) reducing levels, greatly increased (65% and 53%) in this variable was noted at 5 mM CC under stress conditions, respectively. Plants given 10 mM CC accumulated less RS in both varieties under control and stress conditions (Table 4).

Total soluble proteins (TSPs) decreased significantly ($P \leq .001$) in cluster bean plants under salt stress conditions (0 and 150 mM NaCl) as shown in Table 1. Water spray increased the

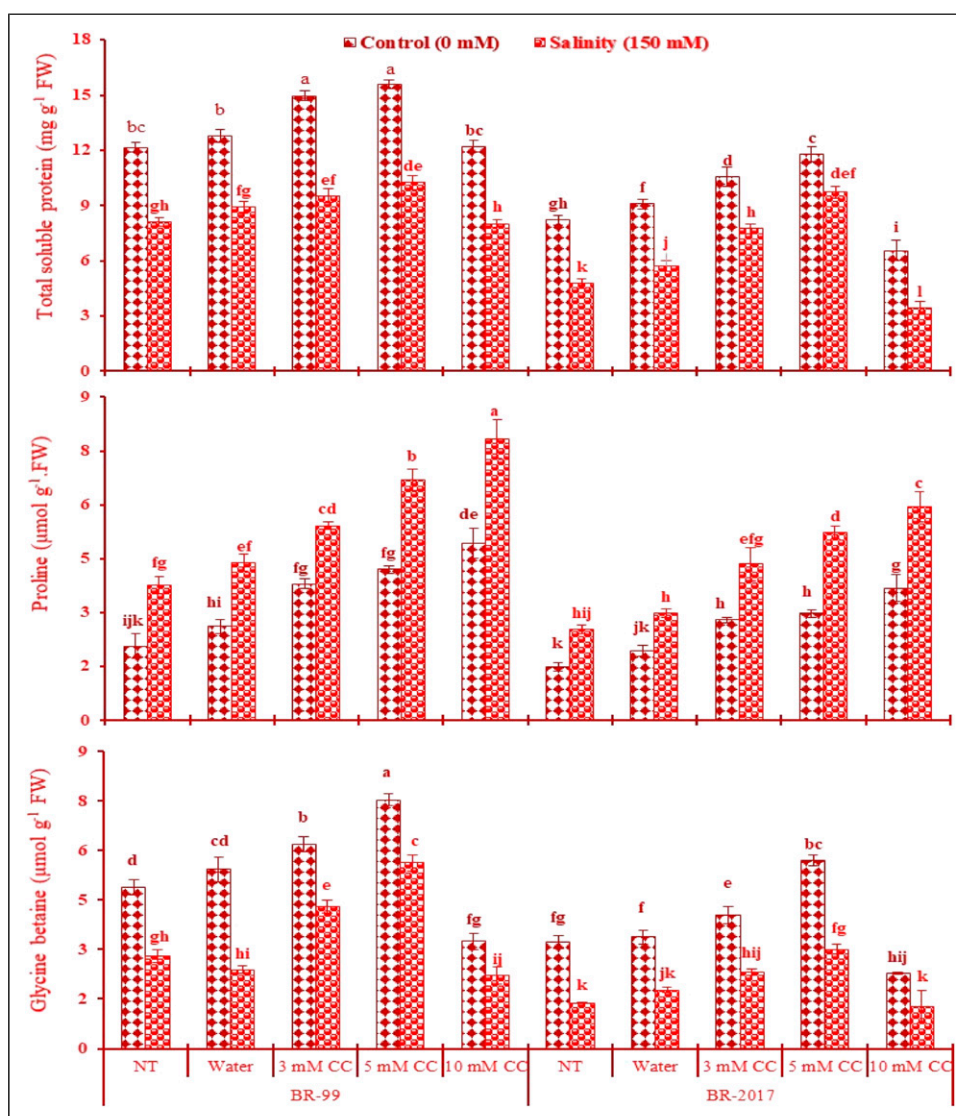


Figure 2. Changes in total soluble protein (TSP), proline contents and glycine betaine (GB) contents in 2 cluster beans varieties sprayed with different regimes of choline chloride (CC) under salt stress ($n = 4 \pm SE$). Bars expressed with different letters are significantly different according to using least significant difference (LSD) at $P \leq .05$.

TSP contents in BR-99 (5% and 10%) and BR-2017 (10% and 20%) under both conditions, respectively. Plants given CC (3 and 5 mM) had even more ($P \leq .001$) TSP in both BR-99 and BR-2017, a higher increase was noted in both BR-99 and BR-2017 at 5 mM CC under normal (28% and 27%) and stressed (44% and 104%) conditions, respectively (Figure 2).

Cluster bean plants had significantly ($P \leq .001$) increased the TFAAs on exposure to stress conditions (Table 1). Decrease in TFAA levels was evident in both BR-99 (40%) and BR-2017 (33%) variety with 150 mM NaCl. In stressed cluster bean plants, CC (3 mM and 5 mM) substantially ($P \leq .001$) improved the TFAA levels (Table 1). Plants given 5 mM CC accumulated more TFAA in BR-99 (50% and 73%) and BR-

2017 (23% and 56%) under control and stress conditions, respectively. Exogenous CC (10 mM) reduced the TFAA contents in both varieties, greatly reduced in BR-2017 (38%) than BR-99 (22%) variety (Table 4).

Proline accumulation was substantial ($P \leq .001$) in cluster bean plants under salt stress conditions (Table 1). Plants grown at 150 mM NaCl level had the greatest increase (82% and 71%) in proline compared with control plants in both BR-99 and BR-2017, respectively. Besides, CC (3, 5, and 10 mM) remarkably enhanced ($P \leq .001$) the proline accumulation in plants under stress conditions (Table 1). Accumulation of proline was the maximum in BR-99 (139% and 148%), and BR-2017 (109% and 134%) varieties administered 10 mM CC

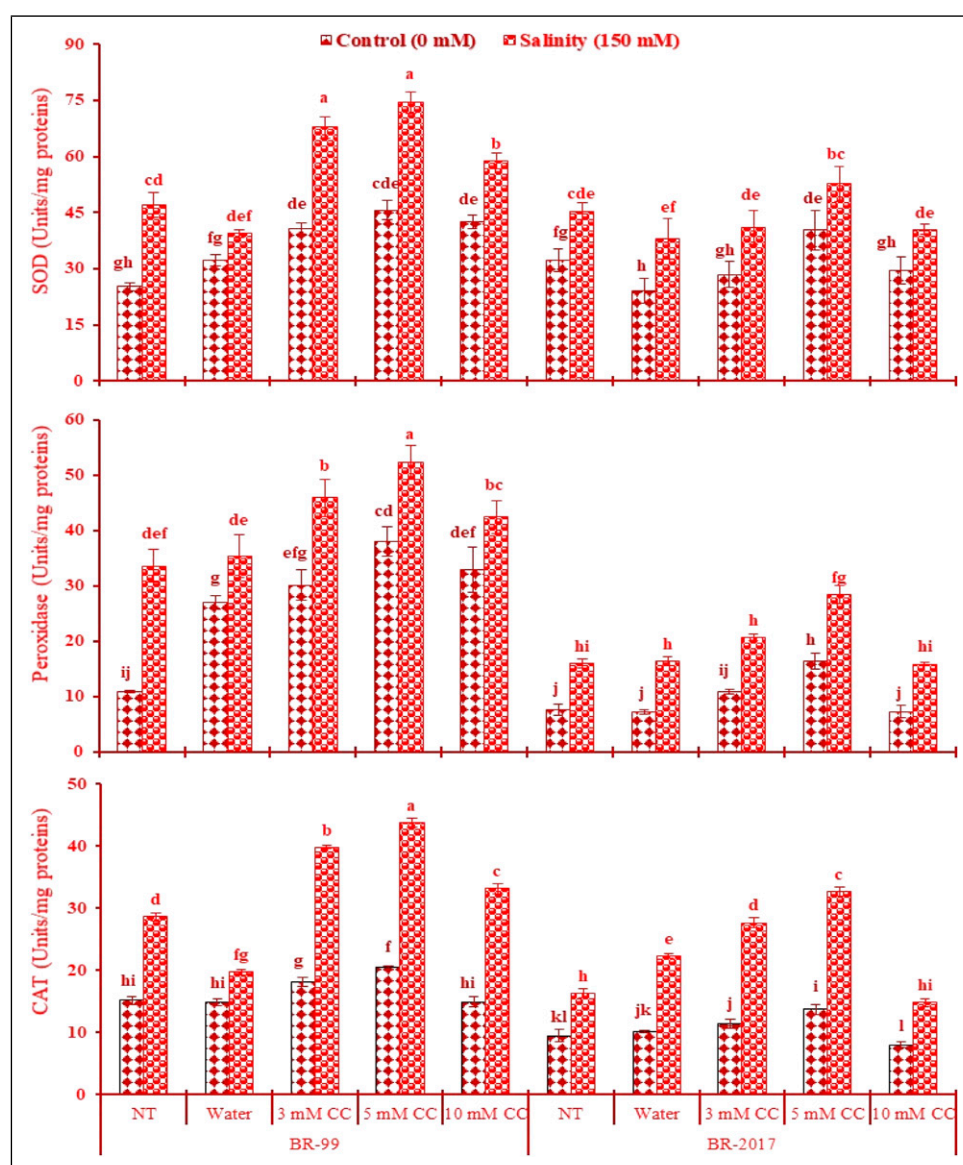


Figure 3. Changes in antioxidant enzymes (SOD, POD and CAT) in 2 cluster beans varieties sprayed with different regimes of choline chloride (CC) under salt stress ($n = 4 \pm SE$). Bars expressed with different letters are significantly different according to using least significant difference (LSD) at $P \leq .05$.

under both control, and stress conditions, respectively (Figure 2).

Exposure of cluster bean plants to salt stress significantly ($P \leq .001$) reduced GB. Plants exposed to 150 mM NaCl had a more drop-in GB content in BR-99 (43%) and BR-2017 (57%). CC (3 and 5 mM) remarkably ($P \leq .001$) improved the GB in both varieties under stress conditions, maximum increase was noted in both BR-99 (54% and 101%) and BR-2017 (78% and 117%) at 5 mM CC under normal and stress conditions, respectively. Plants given 10 mM CC reduced the GB in both varieties under both conditions (Figure 2; Table 1).

Non-Enzymatic Antioxidants. Cluster bean plants had significantly ($P \leq .001$) decreased the Ascorbic acid (ASA) contents when exposed to NaCl stress (Table 1). The lower values for this variable were seen in plants grown during salt stress (150 mM) in BR-99 (16%) and BR-2017 (35%). Moreover, water spray, and CC (3 and 5 mM) administration produced a further increase in ASA content under stress conditions. Under stress, plants fed 5 mM CC indicated a substantial increase in this attribute in BR-99 (127% and 82%) and BR-2017 (133% and 189%) under both conditions, respectively. Furthermore, exogenous 10 mM CC had a significant decrease in ASA levels in BR-99 (56%) and BR-2017 (62%) under stress conditions (Table 4).

Table 1. Mean Sum of Squares from ANOVA of Data for Growth, Physiological and Biochemical Attributes of Cluster Bean Varieties Treated with Choline Chloride (CC) Under Salt Stress.

Source of Variance	Cultivars (Cv), df = 1	Salinity (S) df = 1	Choline Chloride (CC), df = 4	Cv × S, df = 1	Cv × CC, df = 4	S × CC, df = 4	Cv × S × CC, df = 4	Error df = 90
Shoot length	205.922***	518.417***	432.812***	44.477***	66.124***	5.597ns	9.574*	3.798
Root length	48.7006***	85.5361***	58.7181***	6.0051***	3.3786***	1.0118*	.5033ns	.486
Shoot fresh weight	767.802***	470.966***	59.838***	5.222**	6.354***	.888ns	.648ns	1.136
Root fresh weight	354.147***	110.940***	20.177***	15.265***	.633**	1.121***	.865**	.246
Shoot dry weight	22.6731***	10.0587***	8.6817***	.7531***	.0086ns	.0189ns	.315ns	.085
Root dry weight	3.37359***	2.29788***	1.52596***	.20156***	.05770***	.04777**	.00863ns	.012
Chlorophyll a	19.5190***	17.2790***	5.0102***	.5009***	.6340***	.0639***	.0364*	.016
Chlorophyll b	10.7032***	8.1192***	5.5689***	.3814***	.2052***	.0307ns	.0407ns	.025
Total chlorophyll	59.1250***	49.0863***	17.3367***	1.7585***	1.1629***	.11928*	.1044*	.048
Total carotenoids	.49408***	.39889***	.08617***	.02861***	.00407ns	.0038ns	.00244ns	.002
Relative water contents	12 187.0***	6060.6***	2917.0***	265.4***	172.4***	56.5***	4.5ns	7.3
Hydrogen peroxide	389.06***	1089.53***	83.65***	69.53***	1.85ns	10.07***	.10ns	1.86
Malondialdehyde	19.765***	527.014***	47.472***	18.622***	1.750*	14.415***	.636ns	.685
Ascorbic acid	61.0751***	61.4742***	75.0257***	3.8220**	2.3245***	1.7305*	.7246ns	.805
Total phenolic	377.624***	141.601***	37.151***	1.182**	.436ns	.277ns	.131ns	.281
Total flavonoid	204.297***	218.790***	22.029***	5.904***	.539ns	.494ns	.381ns	.443
Anthocyanin	30.2836***	18.8821***	11.4603***	.6423***	.3042***	.1271**	.0498ns	.047
Total free amino acids	46.4281***	53.4056***	9.7976***	5.5799***	.8873***	.3359**ns	.0780	.115
Total soluble protein	243.365***	284.402***	48.921***	13.077***	4.622***	.173ns	1.550***	.345
Proline	27.6307***	69.6060***	26.2162***	1.1010***	.2982ns	1.3005***	.0837ns	.227
Glycine betaine	52.8703***	71.0537***	2 403 059***	.2288ns	1.0880***	1.1360***	.6304***	.150
Total soluble sugar	128 685***	92 672***	99 084***	8ns	5284***	469ns	3245***	637
Reducing sugar	7.17627***	.88364***	2.31093***	1.08783***	.14780*	.14564ns	.12845ns	.072
Superoxide dismutase	2068.20***	5409.17***	906.75***	293.24***	321.43***	71.68*	83.16**	30.27
Peroxidase	9132.87***	2803.83***	624.82***	104.10**	141.20***	33.00*	36.68*	14.21
Catalase	1347.71***	4067.68***	384.94***	85 012***	72.84***	122.98***	47.02***	1.33

***, **, *, significant at .001, .01 and .05 levels, respectively.

Salt stress also caused a significant increase ($P \leq .001$) in anthocyanins in cluster bean plants (Table 1). Plants grown at 150 mM NaCl levels had a maximal decreased in anthocyanin contents in BR-99 (32%) and BR-2017 (28%) compared with control plants. Under salt stress conditions, and CC (3 and 5) treatments to plants raised anthocyanin levels substantially ($P \leq .001$) in both BR-99 and BR-2017 (Table 1). Plants given 5 mM CC accumulated more TFAA when stressed in BR-99 (44% and 70%) and BR-2017 (97% and 125%) under control and stress conditions, respectively (Table 4). Furthermore, exogenous CC (10 mM) reduced the anthocyanin contents in BR-2017 under control (7%) and stress conditions (23%), respectively (Table 4).

A substantial decrease ($P \leq .001$) in phenolic was seen, when cluster bean plants were exposed to NaCl stress (Table 1). In comparison to control plants, plants exposed to 150 mM NaCl level showed less phenolic contents in BR-99 (21%) and BR-2017 (33%). Furthermore, plants given CC (3 and 5 mM) showed greater ($P \leq .001$) phenolic levels under salt stress (Table 1). Under stress conditions, plants fed 5 mM CC showed more phenolic in both BR-99 (19%) and BR-2017 (51%) than control plants. Plants given 10 mM CC reduced the

phenolic contents in BR-2017 (20% and 16%) than BR-99 (13% and 14%) under normal and stress conditions (Table 4). Flavonoids were significantly ($P \leq .001$) decreased in cluster bean plants under salt stress. Plants of both varieties exposed to 150 mM NaCl levels showed the same values for flavonoids. Furthermore, CC (3 and 5 mM) administration improved ($P \leq .001$) flavonoids by several times under stress conditions. Exogenous CC (10 mM) had significantly reduced the flavonoid levels in BR-99 (18% and 9%) and BR-2017 (13% and 14%) under normal and stress conditions, respectively (Table 4).

Antioxidant Enzymes

Salt stress conditions showed an important ($P \leq .001$) increase in the activity of SOD in cluster bean plants. The increase in SOD was visible in BR-99 (87%) and BR-2017 (40%) at 150 mM NaCl. Exogenous application of CC (3, 5 mM) to plants of both varieties, remarkably ($P \leq .001$) improved the SOD activity under salt stress conditions. Plants that have been given 5 mM CC had more improved SOD activity in BR-99 (81% and 58%), and BR-2017 (25% and 16%) under both

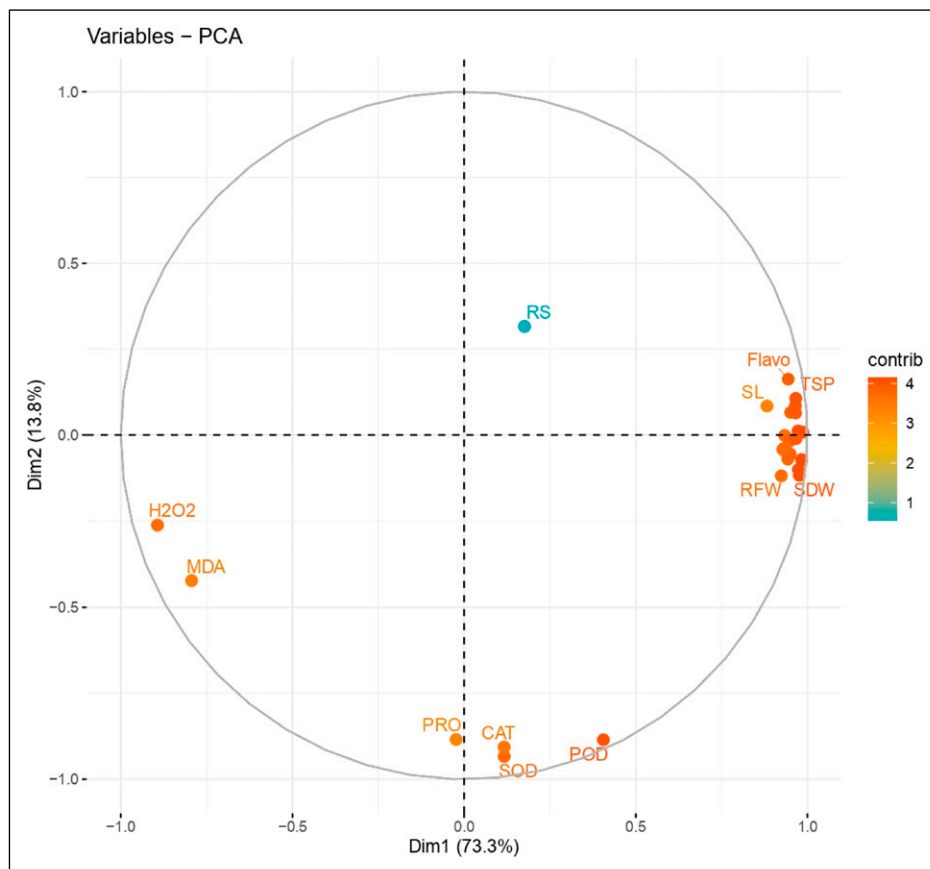


Figure 4. The principal component analysis showing the negative association of oxidative stress markers with the growth attributes and enzymatic antioxidants. Abbreviations: CAT, catalase; Chlb, chlorophyll b; Flavo, flavonoids; GB, glycine betaine; H₂O₂, hydrogen peroxide; MDA, malondialdehyde; POD, peroxidase; PRO, proline; RFW, root fresh weight; RS, reducing sugar; SDW, shoot dry weight; SL, shoot length; SOD, superoxide dismutase; TFAA, total free amino acids; TSP, total soluble protein.

normal and salt stress conditions, respectively. Exogenous CC (10 mM) had reduced the SOD contents in BR-99 (9%) and BR-2017 (11%) under stress (Figure 3; Table 1).

Both varieties of cluster beans showed a substantial ($P \leq .001$) increase in the POD activity when exposed to salt stress. The POD activity of both BR-99 (208%) and BR-2017 (107%) varieties increased under 150 mM NaCl. POD activity rises considerably in both varieties after CC administration (3 and 5 mM). The rise in POD activity was significant in both BR-99 (248% and 55%) and BR-2017 (114% and 78%), when given 5 mM CC under normal and stress conditions. Plants given 10 mM CC increased the POD activity in BR-99 and reduced in BR-2017 under control and salt stress conditions, respectively (Figure 3; Table 1).

Cluster bean displayed a significant ($P \leq .001$) increase in CAT activity when subjected to salt stress. CAT activity was substantially enhanced in plants of both BR-99 (88%) and BR-2017 (71%) grown at 15 mM NaCl. Under stress, CC spray (3 and 5 mM) resulted in a substantial increase in the CAT activity in both BR-99 (39% and 53%) and BR-2017 (70% and 100%), respectively. In this regard, plants given

10 mM CC showed less CAT activity in BR-2017 (15% and 9%) under normal and salt stress conditions, respectively (Figure 3; Table 1).

Principal Component Analysis. The loading plot of the principal component analysis (PCA) was performed to assess the relationship between treatments (control, salt stress, CC, and CC+ salt stress), growth, and physio-biochemical attributes in cluster bean grown in sandy river soil under salinity stress with exogenous applied CC (Figure 4). Among the principal components, components 1 and 2, referred to as Dim1 and Dim2 revealed maximum variation by 71.2% and 13.4%, respectively, with 84.6% of the whole dataset. The first component (PC1) with which the following parameters was positively correlated including growth-related-attributes, chlorophyll contents (Chl.), TFAAs, TSP, GB, POD, SOD, and CAT. A significantly negative correlation of parameters falling in PC1 was observed with the parameters of PC2 such as MDA content (MDA) and hydrogen peroxide (H_2O_2) (Figure 4).

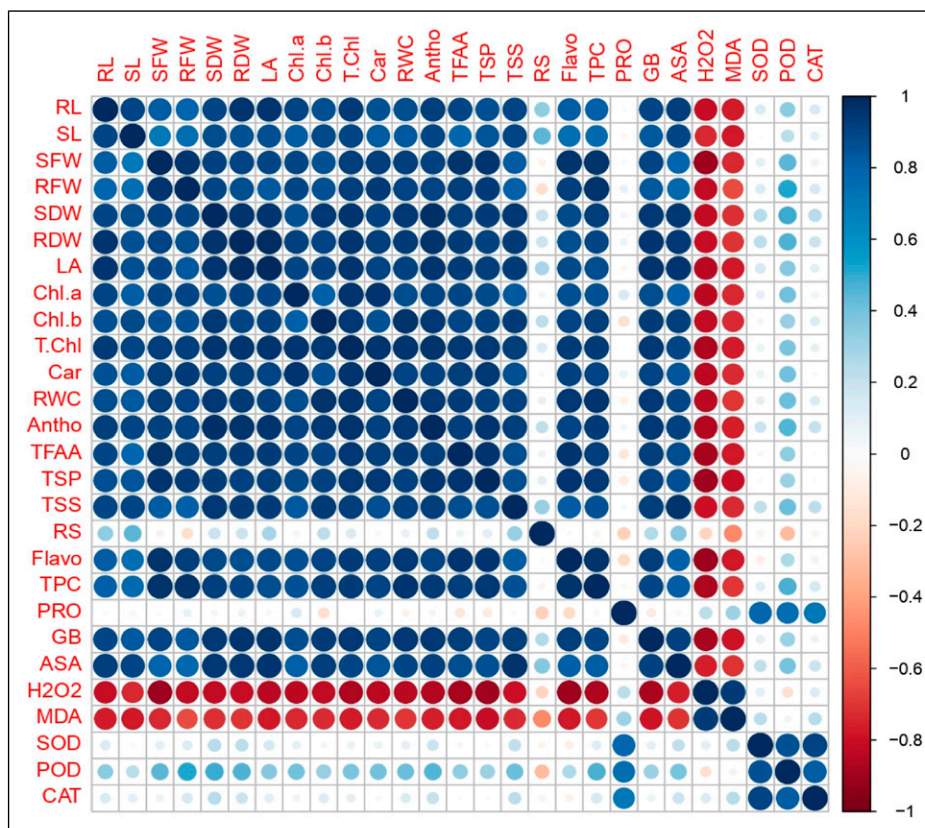


Figure 5. The correlation among different physio-chemical and growth characteristics in cluster bean under salt stress. Abbreviations: Antho, anthocyanin; ASA, ascorbic acid; CAT, catalase; Car, carotenoids; Chl.a, chlorophyll a; Chl.b, chlorophyll b; Flavo, flavonoids; GB, glycine betaine; H_2O_2 , hydrogen peroxide; LA, leaf area; MDA, malondialdehyde; POD, peroxidase; PRO, proline; RDW, root dry weight; RFW, root fresh weight; RL, root length; RS, reducing sugar; RWC, relative water contents; SDW, shoot dry weight; SFW, shoot fresh weight; SL, shoot length; SOD, superoxide dismutase; T.Chl, total chlorophyll; TFAA, total free amino acids; TSP, total soluble protein; TSS, total soluble sugar; TPC, total phenolic contents.

Correlation Matrix. A correlation matrix (Pearson) graph was made to study positive and negative correlations between the studied parameters under the influence of applied treatments and to determine the existing relationships among studied parameters (Figure 5). Salinity treatment was positively correlated with MDA, H₂O₂, Proline, SOD, and CAT content, while negatively correlated with growth attributes, anthocyanin contents, TSP content, chlorophyll (a, b) contents, total chlorophyll, and total carotenoids contents. This association showed a close correlation between antioxidant enzymes and growth in cluster bean (Figure 5).

Discussion

Salinity becomes detrimental to abiotic stress, leading to crop reduction worldwide.^{6,83} The inhibition in plant growth under saline conditions is mainly attributed to Na⁺ toxicity, leads to the failure of normal physio-chemical properties of plants.⁸⁴ As a result, plants with weak roots could not absorb water and nutrients, inhibiting plant development in saline conditions,^{25,85} results in the accumulation of highly reactive oxides in various cells parts, and disturbed the ionic imbalance under saline conditions.^{26,86} The present research results indicated a significant decline in plant growth characteristics

under saline conditions more obvious in BR-2017 than BR-99 on growth variables (Table 2). Usually, salinity above the threshold level of plants lowers the water and beneficial minerals like sodium and calcium uptake leads to plant growth reduction.^{87,88} Exogenous CC (5 mM) resulted in a significant improvement in plant growth characteristics, and photosynthetic pigments in variety BR-2017 as compared to BR-99 (Tables 2 and 3). This counteraction was due to the maintenance of chloroplast membrane,⁸⁹⁻⁹¹ the integrity of thylakoid membrane in photosystem-II,^{92,93} and stability of extrinsic proteins or enzyme-like Rubisco,⁹⁴ under low leaf water potential or high salt concentration. Our findings are consistent with those of Salama et al,⁶⁵ who found a substantial increase in photosynthetic pigments in wheat under salt stress. Choline chloride is an efficient ROS scavenger with the ability to abridge lipid peroxidation and protect membranes. Choline chloride also improves plant growth and development.⁴⁶ Our results demonstrated that a CC-mediated strengthened oxidative defense system efficiently scavenged ROS in both cluster bean varieties, but the most prominent result was seen in the intolerant BR-2017 variety (Fig 1). Choline chloride is also protecting plants from excess ions toxicity and oxidative injury.⁹⁵ Moreover, our study showed that oxidative damage, as assessed by MDA and H₂O₂, greatly hampered plant

Table 2. Effect of Exogenous Choline Chloride (CC) on Growth Related Attributes of 2 Cluster Bean Varieties Grown Under Normal and Salt Stress Conditions.

		SL (cm)	RL (cm)	SFW (g)	SDW (g)	RFW (g)	RDW (g)	LA (cm ²)
BR-99								
Normal	Control	25.50 ^{de} ± 1.80	5.75 ^g ± .55	16.74 ^b ± .75	3.42 ^{de} ± .24	11.16 ^c ± .12	1.18 ^{gh} ± .03	1.28 ^{efg} ± .06
	H ₂ O	27.50 ^{cd} ± .75	7.05 ^{cde} ± .29	17.97 ^b ± .44	3.56 ^{cd} ± .24	12.18 ^b ± .22	1.43 ^{cde} ± .10	1.43 ^{de} ± .10
	CC 3 mM	28.75 ^{bc} ± 1.91	9.25 ^b ± .50	20.68 ^a ± .95	4.15 ^b ± .11	13.41 ^a ± .18	1.84 ^b ± .06	1.91 ^b ± .07
	CC 5 mM	34.00 ^a ± 1.25	11.0 ^a ± .41	21.65 ^a ± 1.01	4.89 ^a ± .12	13.76 ^a ± .19	2.17 ^a ± .11	2.15 ^a ± .04
	CC 10 mM	22.00 ^{gh} ± 1.25	6.80 ^{def} ± .15	17.98 ^b ± .47	3.06 ^{ef} ± .25	11.80 ^{bc} ± .22	1.30 ^{ef} ± .05	1.30 ^{ef} ± .05
	Salinity-150 mM	Control	19.75 ^h ± .99	3.85 ^{hij} ± .74	11.55 ^{ef} ± .53	2.59 ^{gh} ± .12	8.85 ^f ± .58	.95 ^{jk} ± .10
H ₂ O		21.13 ^{gh} ± 1.77	4.15 ^{hi} ± .34	12.78 ^{de} ± .43	2.59 ^{gh} ± .10	9.13 ^{ef} ± .40	.95 ^{jk} ± .06	.95 ^{ji} ± .06
CC 3 mM		22.98 ^{efg} ± 1.68	7.57 ^{cd} ± .44	14.99 ^c ± .59	3.31 ^{def} ± .18	9.80 ^{de} ± .32	1.39 ^{de} ± .06	1.39 ^e ± .06
CC 5 mM		25.24 ^{de} ± .83	8.03 ^c ± .65	17.37 ^b ± .43	3.94 ^{bc} ± .20	10.35 ^d ± .28	1.57 ^c ± .04	1.57 ^{cd} ± .04
CC 10 mM		15.75 ⁱ ± .55	3.18 ^{ij} ± .38	11.52 ^{ef} ± 1.41	2.13 ⁱ ± .31	8.04 ^{gh} ± .27	.87 ^{kl} ± .04	.87 ⁱ ± .04
BR-2017								
Normal	Control	15.25 ^j ± .99	3.69 ^{hij} ± .23	12.07 ^e ± .34	2.23 ^{hi} ± .20	5.93 ^j ± .22	.85 ^{kl} ± .03	1.05 ^{hi} ± .03
	H ₂ O	23.25 ^{efg} ± .99	6.37 ^{efg} ± .56	12.13 ^e ± .23	2.32 ^{hi} ± .15	7.43 ^{hi} ± .16	1.03 ^{hij} ± .03	1.14 ^{gh} ± .03
	CC 3 mM	25.25 ^{de} ± .55	6.93 ^{de} ± .16	13.64 ^{cd} ± .18	2.98 ^{fg} ± .07	8.03 ^{gh} ± .12	1.19 ^{fg} ± .07	1.31 ^{ef} ± .08
	CC 5 mM	31.25 ^{ab} ± .99	7.70 ^{cd} ± .33	14.11 ^{cd} ± .26	3.63 ^{cd} ± .06	8.57 ^{fg} ± .23	1.52 ^{cd} ± .10	1.68 ^c ± .11
	CC 10 mM	19.25 ^{hi} ± .87	4.61 ^h ± .40	9.53 ^g ± .21	1.63 ^j ± .05	6.93 ⁱ ± .16	.77 ^{lm} ± .08	.85 ^{jk} ± .08
Salinity-150 mM	Control	10.75 ^k ± .73	1.97 ^k ± .08	7.48 ^h ± .66	1.64 ^j ± .170	4.45 ^k ± .38	.67 ^m ± .04	.70 ^{kl} ± .05
	H ₂ O	16.50 ^{ji} ± .33	4.55 ^h ± .30	7.67 ^h ± .53	1.67 ^j ± .16	5.75 ^j ± .17	.76 ^{lm} ± .04	.80 ^{kl} ± .05
	CC 3 mM	24.50 ^{ef} ± .33	5.92 ^{fg} ± .26	8.91 ^{gh} ± .70	2.37 ^{hi} ± .16	6.98 ⁱ ± .21	1.00 ^{ijk} ± .07	1.05 ^{hi} ± .07
	CC 5 mM	29.75 ^{bc} ± 1.28	6.32 ^{efg} ± .40	10.24 ^{fg} ± .42	3.22 ^{def} ± .08	8.07 ^{gh} ± .29	1.12 ^{ghi} ± .02	1.18 ^{gh} ± .02
	CC 10 mM	14.75 ^j ± .73	2.94 ^{jk} ± .13	5.49 ⁱ ± .13	1.32 ^j ± .14	4.25 ^k ± .50	.62 ^m ± .05	.65 ^l ± .05

Means are followed by standard errors. Means were compared with least significance difference (LSD ≤ .05) and different letters indicate that means are different at 95% confidence level. Abbreviations: LA, leaf area; RDW, root dry weight; RFW, root fresh weight; RL, root length; SDW, shoot dry weight; SFW, shoot fresh weight; SL, shoot length.

growth (Figure 4). Furthermore, growth-related attributes (SL, RL, SFW, RFW, SDW, RDW, and LA), physiological attributes (Chl. a Chl. b, total Chl. total carotenoid, and RWC), and biochemical attributes (anthocyanin, TFAA, TSP, TSS, RS, flavonoid, phenolics, and GB) were negatively correlated with oxidative stress markers such as H₂O₂, and MDA levels (Figure 5). Choline is the precursor of phosphatidylcholine, which ensures protection for membrane fluidity and permeability in the membrane transport system.⁹⁵ Our results coincide with the findings of Sakamoto and Murata⁹⁶ and Salama et al,⁶⁵ who found a significant decrease in lipid peroxidation. Salt stress induces significant injury to membranes, as evident in the form of higher electrolyte leakage (EL) and greater lipid peroxidation.⁹⁷ As a result, the decrease in RWC and enhancement in EL is estimated as plasma membranes cannot efficiently regulate the movement of substances.⁹⁸ The decrease in RWC was also seen in *Amaranthus tricolor* plants under salt stress.⁹⁹ Odjegba and Chukwunwike¹⁰⁰ reported a decrease in RWC contents in *Amaranthus hybridus* plants under saline conditions. The inorganic ions and organic metabolites, including TFAAs, TSS, and RS are involved in osmoregulation.⁶ Choline chloride improved ROS detoxification; results in a decrease in lipid peroxidation (MDA) and relative membrane

permeability.⁹⁵ Moreover, plants treated with CC in saline conditions showed higher osmotic adjustment, as seen by improvement in several compatible solutes accumulation in cytoplasm, which might have increased the RWC in plants.¹⁰¹

Chlorophyll pigment is a potential indicator to determine the photosynthetic capacity of plants under saline conditions.¹⁰² Salt stress hampered the chlorophyll synthesis and enhanced the pigment degradation, in addition to more chloroplast structural decline.¹⁰³ The deterioration in chlorophyll pigments led to reduced plant biomass under saline conditions.¹⁹ This study also revealed that plants with the maximum chlorophyll molecule breakdown in saline conditions showed minimal plant biomass (Table 2). The breakdown of chlorophyll molecules is also greater in salt stress conditions due to the greater activity of chlorophyllase.¹⁰⁴ Rasheed et al²² indicated a significant degradation of chlorophyll molecules in sunflower plants under salt stress conditions. Plants have given CC-mediated better ROS detoxification and dropped the breakdown of chlorophyll under stress conditions.³⁸

The increased production of osmolytes like TFAA, TSS, TSP, proline, and GB remarkably reduced the osmotic potential of the cell. The decrease in osmotic potential is helpful for water intake and maintenance of cell turgidity. Therefore, greater levels of osmolytes also defended the plants from ions

Table 3. Effect of Exogenous Choline Chloride (CC) on Photosynthetic Pigments and Relative Water Contents of 2 Cluster Bean Varieties Grown Under Normal and Salt Stress Conditions.

		Chl. a (mg g ⁻¹ FW)	Chl. b (mg g ⁻¹ FW)	T.Chl (mg g ⁻¹ FW)	Total Car. (mg g ⁻¹ FW)	RWC (%)
BR-99						
Normal	Control	3.78 ^e ± .06	3.07 ^c ± .10	6.85 ^d ± .14	.51 ^{de} ± .01	62.99 ^d ± 1.71
	H ₂ O	4.15 ^c ± .02	3.06 ^c ± .18	7.21 ^c ± .16	.60 ^{bc} ± .03	72.05 ^c ± 1.60
	CC 3 mM	4.73 ^b ± .15	3.48 ^b ± .12	8.21 ^b ± .23	.67 ^b ± .06	77.51 ^b ± 2.37
	CC 5 mM	5.58 ^a ± .06	3.76 ^a ± .09	9.34 ^a ± .11	.74 ^a ± .05	88.16 ^a ± 2.49
	CC 10 mM	4.25 ^c ± .06	2.00 ^{hi} ± .06	6.26 ^e ± .10	.56 ^{cd} ± .04	41.25 ^g ± 1.29
	Salinity-150 mM	Control	3.01 ^h ± .09	2.29 ^{ef} ± .10	5.30 ^{fg} ± .12	.51 ^{de} ± .01
H ₂ O		3.09 ^h ± .08	2.35 ^{def} ± .08	5.44 ^f ± .09	.42 ^{gh} ± .05	49.33 ^f ± 2.48
CC 3 mM		3.48 ^f ± .09	2.49 ^{de} ± .03	5.96 ^e ± .11	.46 ^{efg} ± .02	57.92 ^e ± 1.89
CC 5 mM		4.33 ^c ± .08	2.89 ^c ± .05	7.21 ^c ± .12	.50 ^{def} ± .02	63.45 ^d ± .95
CC 10 mM		3.15 ^{gh} ± .04	1.48 ^j ± .08	4.63 ^{ij} ± .11	.40 ^{gh} ± .01	26.13 ⁱ ± 1.32
BR-2017						
Normal	Control	2.39 ^j ± .15	1.84 ⁱ ± .07	4.23 ^k ± .16	0.3 ^{jk} ± .02	31.51 ^h ± 1.39
	H ₂ O	3.31 ^{fg} ± .05	2.23 ^{fg} ± .23	5.54 ^f ± .27	.40 ^{ghi} ± .01	42.60 ^g ± 1.75
	CC 3 mM	3.70 ^e ± .02	2.53 ^d ± .03	6.22 ^e ± .04	.44 ^{fgh} ± .02	47.85 ^f ± .74
	CC 5 mM	3.97 ^d ± .04	2.99 ^c ± .05	6.95 ^{cd} ± .06	.55 ^{cd} ± .01	54.32 ^e ± 1.33
	CC 10 mM	3.40 ^f ± .04	1.43 ^j ± .05	4.83 ^{hi} ± .08	.38 ^{hij} ± .01	24.04 ⁱ ± 1.05
	Salinity-150 mM	Control	1.65 ^k ± .08	1.26 ^j ± .03	2.91 ^m ± .11	.22 ^l ± .02
H ₂ O		2.57 ^{ij} ± .05	1.81 ⁱ ± .05	4.39 ^{jk} ± .09	.28 ^{kl} ± .01	25.76 ^j ± 1.19
CC 3 mM		2.99 ^h ± .06	2.07 ^{gh} ± .03	5.06 ^{gh} ± .05	.31 ^k ± .01	32.91 ^h ± 1.02
CC 5 mM		3.01 ^h ± .02	2.48 ^{de} ± .04	5.48 ^f ± .03	.45 ^{efg} ± .01	39.73 ^g ± 1.56
CC 10 mM		2.69 ⁱ ± .07	.90 ^k ± .04	3.60 ^l ± .09	.33 ^{ijk} ± .02	17.28 ^j ± .55

Means are followed by standard errors. Means were compared with least significance difference (LSD ≤ .05) and different letters indicate that means are different at 95% confidence level. Abbreviations: Chl. a, chlorophyll a; Chl. b, chlorophyll b; RWC, relative water content; Total Car., total carotenoids; Total Chl., total chlorophyll.

Table 4. Effect of Exogenous Choline Chloride (CC) on Biochemical Attributes of 2 Cluster Bean Varieties Grown Under Normal and Salt Stress Conditions.

		Ascorbic Acid (mg g ⁻¹ FW)	Phenolic (mg g ⁻¹ FW)	Flavonoids (mg g ⁻¹ FW)	Anthocyanin (Unit g ⁻¹ FW)	TFAA (mg g ⁻¹ FW)	TSS (mg g ⁻¹ FW)	Reducing Sugar (mg g ⁻¹ FW)	
BR-99	Normal	Control	4.48 ^g ± .15	13.88 ^b ± .35	12.93 ^b ± .29	3.84 ^{de} ± .14	4.57 ^{de} ± .20	285.20 ^{fg} ± 6.26	1.72 ^{ghij} ± .08
		H ₂ O	6.20 ^{de} ± .56	14.11 ^b ± .19	13.03 ^b ± .50	4.32 ^c ± .09	5.31 ^c ± .19	337.35 ^{de} ± 20.57	1.86 ^{gh} ± .08
		CC 3 mM	8.70 ^b ± .55	15.32 ^a ± .28	14.13 ^a ± .52	4.80 ^b ± .12	6.22 ^b ± .29	421.24 ^b ± 12.38	1.77 ^{ghij} ± .14
		CC 5 mM	10.20 ^a ± .3	15.82 ^a ± .49	14.64 ^a ± .64	5.51 ^a ± .16	6.83 ^a ± .08	457.29 ^a ± 16.57	1.94 ^g ± .19
		CC 10 mM	4.38 ^{fg} ± .44	12.01 ^d ± .39	10.60 ^c ± .45	3.58 ^{ef} ± .14	4.83 ^{cd} ± .26	219.09 ^{hi} ± 15.31	1.45 ^{ij} ± .13
	Salinity-150 mM	Control	3.77 ^{gh} ± .93	10.92 ^e ± .23	8.50 ^{gh} ± .24	2.60 ⁱ ± .12	2.74 ^{kl} ± .27	246.85 ^h ± 12.05	1.42 ^j ± .13
		H ₂ O	4.30 ^{fg} ± .94	11.00 ^e ± .21	9.22 ^{efg} ± .36	2.83 ^{hi} ± .09	3.20 ^{hij} ± .22	253.87 ^{gh} ± 11.46	1.54 ^{hij} ± .10
		CC 3 mM	6.40 ^{de} ± .22	12.32 ^{cd} ± .3	10.06 ^{cde} ± .3	3.69 ^{ef} ± .08	4.11 ^{ef} ± .18	324.60 ^e ± 20.23	2.14 ^{def} ± .15
		CC 5 mM	6.88 ^{cd} ± .32	12.96 ^c ± .35	10.50 ^{cd} ± .5	4.42 ^c ± .15	4.76 ^d ± .11	376.26 ^c ± 14.04	2.35 ^{de} ± .12
		CC 10 mM	1.65 ^{jk} ± .22	9.44 ^{fg} ± .33	7.77 ^{hi} ± .39	2.75 ⁱ ± .13	2.15 ⁿ ± .18	175.10 ⁱ ± 10.52	1.40 ^j ± .07
BR-2017	Normal	Control	3.43 ^{ghi} ± .20	9.08 ^{fg} ± .20	9.01 ^{fg} ± .25	2.10 ^j ± .15	3.28 ^{hi} ± .15	225.69 ^{hi} ± 11.73	2.32 ^{de} ± .13
		H ₂ O	3.53 ^{gh} ± .35	9.76 ^f ± .22	9.61 ^{def} ± .24	3.12 ^{cd} ± .07	3.49 ^{gh} ± .13	197.66 ^{ij} ± 14.21	2.41 ^{cd} ± .13
		CC 3 mM	5.18 ^{ef} ± .65	10.86 ^e ± .23	9.78 ^{cdef} ± .35	3.69 ^{ef} ± .16	3.84 ^{fg} ± .18	302.76 ^{ef} ± 17.26	2.85 ^b ± .20
		CC 5 mM	8.02 ^{bc} ± .55	11.23 ^e ± .20	10.40 ^{cd} ± .57	4.13 ^{cd} ± .18	4.03 ^f ± .24	362.57 ^{cd} ± 17.29	3.30 ^a ± .16
		CC 10 mM	2.88 ^{hij} ± .54	7.28 ^h ± .31	7.82 ^{hi} ± .26	1.94 ^j ± .11	2.87 ^{ijk} ± .35	227.27 ^{hi} ± 20.11	2.02 ^{efg} ± .20
	Salinity-150 mM	Control	2.23 ^{ij} ± .51	6.07 ⁱ ± .28	5.87 ^k ± .29	1.52 ^k ± .17	2.20 ^{mn} ± .08	123.91 ^k ± 8.44	1.81 ^{ghi} ± .22
		H ₂ O	2.91 ^{hij} ± .58	7.10 ^h ± .30	6.82 ^j ± .30	2.08 ^j ± .05	2.37 ^{mn} ± .19	172.23 ^j ± 8.49	1.88 ^{gh} ± .24
		CC 3 mM	3.99 ^{gh} ± .26	8.72 ^g ± .39	7.12 ^{ij} ± .34	2.84 ^{hi} ± .12	2.63 ^{km} ± .14	234.15 ^h ± 15.94	2.45 ^{cd} ± .21
		CC 5 mM	6.47 ^d ± .84	9.14 ^{fg} ± .38	7.92 ^{hi} ± .27	3.42 ^{fg} ± .11	3.43 ^{gh} ± .11	321.71 ^e ± 19.80	2.77 ^{bc} ± .15
		CC 10 mM	.86 ^k ± .03	5.09 ^j ± .32	5.06 ^k ± .31	1.17 ^j ± .08	1.36 ^o ± .08	126.76 ^k ± 3.03	1.76 ^{ghij} ± .10

Means are followed by standard errors. Means were compared with least significance difference (LSD ≤ .05) and different letters indicate that means are different at 95% confidence level. Abbreviations: TFAA, total free amino acids; TSS, total soluble sugar.

excess toxicity under saline conditions.²² The results of this study indicated that there was a significant increase in osmolytes such as proline, proteins, and GB in cluster bean plants when subjected to salt stress conditions (Figure 2). Rasheed et al.²² also reported same results in sunflowers under salt stress. Total soluble sugars and GB contents showed a positive association with the growth of cluster bean plants under saline conditions (Figure 5). Choline chloride-mediated enhancement in these compatible solutes in the cytoplasm results in considerably increased plant growth under salt stress conditions. This GB production was likened to the metabolism of CC into GB.^{65,105} Phenolic compounds are strong antioxidants that efficiently hunt the ROS, and reduce the lipids peroxidation in *Phaseolus vulgaris* plants, consequently, phenolic protects the plants against oxidative injury.¹⁰⁶ Higher accumulation of phenolic shows a greater stress tolerance level.^{107,108} Under salt stress, CC significantly increases the phenolic contents in cluster bean (Table 4). Our findings are in line with the results of similar responses reported in potato.¹⁰⁹ Likewise, anthocyanins not only detoxify the free radicals but nevertheless, anthocyanins also inhibit them from generating in stressed plants. Results of this study showed that plants treated with CC produced a significant rise in anthocyanins contents under salt stress conditions (Table 4). In previous literature, exogenous CC improved the growth of radish plants with an increase in phenylalanine, anthocyanin.¹⁰⁹ Flavonoids are antioxidants that help in the regulation of enzyme activity and the production of primary metabolites. Flavonoids accumulate in plant tissues and have significant potential to scavenge the free radicals species.^{110,111} Flavonoids defend the plants from oxidative damage,¹¹² and plants with lesser oxidative impairment depict greater plant growth under salt stress conditions.¹¹³ Plants treated with CC showed higher flavonoids that improved the salt stress tolerance in cluster bean plants (Table 4).

Ascorbic acid and TSP accumulate in the tissues of plants and have a strong potential to scavenge the free radicals.^{114,115} Several studies in the literature have shown that stability of protein and its assemblage under stress is necessary to maintain membrane integrity and cellular persistence in tomato,¹¹⁶ *Vicia faba*,¹¹⁷ and maize¹¹⁸ though the decline in protein accumulation in plants under salinity is also reported.¹¹⁹ In this study, our results are consistent with those of Khurana et al.,⁹⁰ who found a substantial decrease in TSP under salt stress. Similarly, ascorbic acid contents were reduced under salt stress (Table 4).

Salt stress causes a substantial change in antioxidant enzyme activities. The higher antioxidant enzyme activities powerfully protected the plants from oxidative damage reflected as insignificant levels for MDA, and H₂O₂, contents in plants.^{120,121} In this study, a significant rise in oxidative damage revealed a greater free radicals generation, loss of membrane integrity, and enhanced lipid peroxidation under salt stress conditions (Figure 1) Furthermore, we observed that H₂O₂ showed a positive correlation with CAT and SOD

activity. By contrast, POD had a substantial negative association with H₂O₂, and reducing sugar (Figure 5). Choline chloride supplementation in plants enhanced the antioxidant compounds and antioxidant enzyme activities that lessened the oxidative damage reflected as lesser MDA and H₂O₂ contents (Figure 3).

Conclusions

Salt stress considerably decreased the growth characteristics, and photosynthetic pigments, in cluster bean plants. In addition, MDA and H₂O₂ production were several folds greater in plants under salt stress. Variety BR-2017 was found to be sensitive, contained a higher concentration of oxidative stress markers (H₂O₂ and MDA), under salt stress. Choline chloride supplementation resulted in a substantial increase in growth-related attributes, chlorophyll pigments, and antioxidants potential. Choline chloride enhanced free radical detoxification and substantially protected the plants from oxidative impairment under salt stress. Furthermore, CC protected the plants from osmotic stress under salt stress conditions. Choline chloride (5 mM) has shown a significant potential to increase more tolerance to salt stress by regulating the growth, physiological and biochemical, and antioxidant attributes in cluster bean. Thus, this study showed that the CC application (5 mM) is an efficient strategy for field use in the areas, where salt stress soils limit agriculture production.

Acknowledgments

The presented data is a part of PhD dissertation of MS. Saima Riaz and financially supported by GCUF-RSP (4325-4359) and Project Code: 48-BOT-7.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

ORCID iD

Iqbal Hussain  <https://orcid.org/0000-0002-8449-0494>

References

1. Abdelaal KA, EL-Maghraby LM, Elansary H, Hafez YM, Ibrahim EI, El-Banna M, et al. Treatment of sweet pepper with stress tolerance-inducing compounds alleviates salinity stress oxidative damage by mediating the physio-biochemical activities and antioxidant systems. *Agronomy*. 2020;10(1):26.
2. Akbar A, Ashraf MA, Rasheed R, Ali S, Rizwan M Menadione sodium bisulphite regulates physiological and biochemical

- responses to lessen salinity effects on wheat (*Triticum aestivum* L.). *Physiol Mol Biol Plants*. 2021;27(5):1135-1152.
3. Dutta T, Neelapu NRR, Wani SH, Challa S. Compatible solute engineering of crop plants for improved tolerance toward abiotic stresses. *Biochemical, Physiological and Molecular Avenues for Combating Abiotic Stress Tolerance in Plants*. Amsterdam, The Netherlands: Elsevier; 2018:221-254.
 4. Ghafoor A, Qadir M, Murtaza G. *Salt-Affected Soils: Principles of Management*. Urdu Bazar, Lahore: Allied Book Centre; 2004.
 5. Munns R, Tester M. Mechanisms of salinity tolerance. *Annu Rev Plant Biol*. 2008;59:651-681.
 6. Wang P, Zhao Y, Li Z, Hsu C-C, Liu X, Fu L, et al. Reciprocal regulation of the TOR kinase and ABA receptor balances plant growth and stress response. *Mol Cell*. 2018;69(1): 100-112.
 7. Bordenave CD, Rocco R, Maiale SJ, Campestre MP, Ruiz OA, Rodríguez AA, et al. Chlorophyll a fluorescence analysis reveals divergent photosystem II responses to saline, alkaline and saline-alkaline stresses in the two *Lotus japonicus* model ecotypes MG20 and Gifu-129. *Acta Physiol Plant*. 2019;41(9):167.
 8. Xie Z, Song R, Shao H, Song F, Xu H, Lu Y. Silicon improves maize photosynthesis in saline-alkaline soils. *Scientific World J*. 2015;2015:245072.
 9. Flörke M, Bärlund I, van Vliet MT, Bouwman AF, Wada Y. Analysing trade-offs between SDGs related to water quality using salinity as a marker. *Curr Opin Environ Sustain*. 2019;36: 96-104.
 10. Huang M, Chai L, Jiang D, Zhang M, Zhao Y, Huang Y. Increasing aridity affects soil archaeal communities by mediating soil niches in semi-arid regions. *Sci Total Environ*. 2019;647: 699-707.
 11. Sui N, Tian S, Wang W, Wang M, Fan H. Over expression of glycerol-3-phosphate acyltransferase from *Suaeda salsa* improves salt tolerance in *Arabidopsis*. *Front Plant Sci*. 2017;8: 1337.
 12. Bai J, Qin Y, Liu J, Wang Y, Sa R, Zhang N, et al. Proteomic response of oat leaves to long-term salinity stress. *Environ Sci Pollut Res Int*. 2017;24(4):3387-3399.
 13. Bybordi A. The influence of salt stress on seed germination, growth and yield of canola cultivars. *Not Bot Horti Agrobo*. 2010;38(1):128-133.
 14. Sanchez PL, Chen M-k, Pessaraki M, Hill HJ, Gore MA, Jenks MA. Effects of temperature and salinity on germination of non-pelleted and pelleted guayule (*Parthenium argentatum* A. Gray) seeds. *Ind Crop Prod*. 2014;55:90-96.
 15. Acquaaah G. *Principles of Plant Genetics and Breeding*. Hoboken: John Wiley and Sons; 2009.
 16. Ahmad M, Shahzad A, Iqbal M, Asif M, Hirani AH. Morphological and molecular genetic variation in wheat for salinity tolerance at germination and early seedling stage. *Aust J Crop Sci*. 2013;7(1):66-74.
 17. Khan MA, Shirazi MU, Khan MA, Mujtaba SM, Islam E, Mumtaz S, et al. Role of proline, K/Na ratio and chlorophyll content in salt tolerance of wheat (*Triticum aestivum* L.). *Pakistan J Bot*. 2009;41(2):633-638.
 18. Qadir M, Quillérou E, Nangia V, Murtaza G, Singh M, Thomas RJ, et al. Economics of salt-induced land degradation and restoration. *Nat Resour Forum*. 2014;38:282-295.
 19. Hussain I, Ashraf MA, Anwar F, Rasheed R, Niaz M, Wahid A. Biochemical characterization of maize (*Zea mays*L.) for salt tolerance. *Plant Biosyst*. 2014;148(5):1016-1026.
 20. Rasheed R, Ashraf MA, Parveen S, Iqbal M, Hussain I. Effect of salt stress on different growth and biochemical attributes in two canola (*Brassica napus* L.) cultivars. *Commun Soil Sci Plant Anal*. 2014;45(5):669-679.
 21. Babar S, Siddiqi EH, Hussain I, Hayat Bhatti K, Rasheed R. Mitigating the effects of salinity by foliar application of salicylic acid in Fenugreek. *Physiol J*, 2014;2014:1-6.
 22. Rasheed R, Parveen A, Riaz M, Arif MS, Hussain I, Firdous S, et al. Soil biochar ameliorates salinity stress and improves nutrient uptake, biomass production and physiochemical parameters in sunflower. *Inter J Agric Biol*. 2019;22(6): 1663-1674.
 23. Gilliham M, Able JA, Roy SJ. Translating knowledge about abiotic stress tolerance to breeding programmes. *Plant J*. 2017; 90(5):898-917.
 24. Zhang H, Liu XL, Zhang RX, Yuan HY, Wang MM, Yang HY, et al. Root damage under alkaline stress is associated with reactive oxygen species accumulation in rice (*Oryza sativa* L.). *Front Plant Sci*. 2017;8(1580):1580.
 25. Bose J, Munns R, Shabala S, Gilliham M, Pogson B, Tyerman SD. Chloroplast function and ion regulation in plants growing on saline soils: Lessons from halophytes. *J Exp Bot*. 2017; 68(12):3129-3143.
 26. Shabala S, White RG, Djordjevic MA, Ruan Y-L, Mathesius U. Root-to-shoot signalling: Integration of diverse molecules, pathways and functions. *Funct Plant Biol*. 2016;43(2):87-104.
 27. Jin X, Liu T, Xu J, Gao Z, Hu X. Exogenous GABA enhances muskmelon tolerance to salinity-alkalinity stress by regulating redox balance and chlorophyll biosynthesis. *BMC Plant Biol*. 2019;19(1):48-15.
 28. Wu H, Zhang X, Giraldo JP, Shabala S. It is not all about sodium: Revealing tissue specificity and signalling roles of potassium in plant responses to salt stress. *Plant Soil*. 2018; 431(1):1-17.
 29. Parida A, Das A, Mitra B. Effects of NaCl stress on the structure, pigment complex composition, and photosynthetic activity of mangrove *Bruguiera parviflora* chloroplasts. *Photosynthetica*. 2003;41(2):191-200.
 30. Rasheed R, Arslan Ashraf M, Kamran S, Iqbal M, Hussain I. Menadione sodium bisulphite mediated growth, secondary metabolism, nutrient uptake and oxidative defense in okra (*Abelmoschus esculentus* Moench) under cadmium stress. *J Hazard Mater*. 2018;360:604-614.
 31. Singh PK, Singh R, Singh S. Cinnamic acid induced changes in reactive oxygen species scavenging enzymes and protein profile in maize (*Zea mays* L.) plants grown under salt stress. *Physiol Mol Biol Plants*. 2013;19(1):53-59.
 32. Fu J, Liu Z, Li Z, Wang Y, Yang K. Alleviation of the effects of saline-alkaline stress on maize seedlings by regulation of active

- oxygen metabolism by *Trichoderma asperellum*. *PLoS One*. 2017;12(6):e0179617.
33. Lv BS, Li XW, Ma HY, Sun Y, Wei LX, Jiang CJ, et al. Differences in growth and physiology of rice in response to different saline-alkaline stress factors. *Agron J*. 2013;105(4):1119-1128.
 34. Wang H-L, Wang L, Tian C-Y, Huang Z-Y. Germination dimorphism in *Suaeda acuminata*: A new combination of dormancy types for heteromorphic seeds. *S Afri J Bot*. 2012;78:270-275.
 35. Kasote DM, Katyare SS, Hegde MV, Bae H. Significance of antioxidant potential of plants and its relevance to therapeutic applications. *Int J Biol Sci*. 2015;11(8):982-991.
 36. Mansour MMF, Ali EF. Evaluation of proline functions in saline conditions. *Phytochemistry*. 2017;140:52-68.
 37. Chin EWM, Lim WM, Ma D, Rosales FJ, Goh ELK. Choline rescues behavioral deficits in a mouse model of Rett syndrome by modulating neuronal plasticity. *Mol Neurobiol*. 2019;56(6):3882-3896.
 38. Hussain I, Saleem MH, Mumtaz S, Rasheed R, Ashraf MA, Maqsood F, et al. Choline chloride mediates chromium tolerance in spinach (*Spinacia oleracea* L.) by restricting its uptake in relation to morpho-physio-biochemical Attributes. *J Plant Growth Regul* 2021;1-21. doi:10.1007/s00344-021-10401-7.
 39. Zheng R, Chen G, Li J, Wang C. Choline chloride treatments promote ornamental quality of Liliium oriental hybrids' sorbonne. Paper presented at the XXIX international horticultural congress on horticulture: sustaining lives, livelihoods and landscapes (IHC2014); 2014:123-126.
 40. Rathinasabapathi B, McCue KF, Gage DA, Hanson AD. Metabolic engineering of glycine betaine synthesis: Plant betaine aldehyde dehydrogenases lacking typical transit peptides are targeted to tobacco chloroplasts where they confer betaine aldehyde resistance. *Planta*. 1994;193(2):155-162.
 41. Sanchez-Lopez E, Zhong Z, Stubelius A, Sweeney SR, Booshehri LM, Antonucci L, et al. Choline uptake and metabolism modulate macrophage IL-1 β and IL-18 production. *Cell Metabol*. 2019;29(6):1350-1362.
 42. Ifuku K, Noguchi T. Structural coupling of extrinsic proteins with the oxygen-evolving center in photosystem II. *Front Plant Sci*. 2016;7:84.
 43. Sasi S, Venkatesh J, Daneshi R, Gururani M. Photosystem II extrinsic proteins and their putative role in abiotic stress tolerance in higher plants. *Plants*. 2018;7(4):100.
 44. Mansour MMF, Salama KHA, Al-Mutawa MM. Transport proteins and salt tolerance in plants. *Plant Sci*. 2003;164(6):891-900.
 45. Hanson AD, Rathinasabapathi B, Chamberlin B, Gage DA. Comparative physiological evidence that β -alanine betaine and choline-O-sulfate act as compatible osmolytes in halophytic *Limonium* species. *Plant Physiol*. 1991;97(3):1199-1205.
 46. McNeil SD, Nuccio ML, Ziemak MJ, Hanson AD. Enhanced synthesis of choline and glycine betaine in transgenic tobacco plants that overexpress phosphoethanolamine N-methyltransferase. *Proc Natl Acad Sci U S A*. 2001;98(17):10001-10005.
 47. Pavić V, Flačer D, Jakovljević M, Molnar M, Jokić S. Assessment of total phenolic content, in vitro antioxidant and antibacterial activity of *Ruta graveolens* L. extracts obtained by choline chloride based natural deep eutectic solvents. *Plants*. 2019;8(3):69.
 48. Radošević K, Bubalo MC, Srček VG, Grgas D, Dragičević TL, Redovniković IR. Evaluation of toxicity and biodegradability of choline chloride based deep eutectic solvents. *Ecotoxicol Environ Saf*. 2015;112:46-53.
 49. Yuniarti E, Saputri FC, Mun'im A. Application of the natural deep eutectic solvent choline chloride-sorbitol to extract chlorogenic acid and caffeine from green coffee beans (*Coffea canephora*). *J Appl Pharm Sci*. 2019;9(3):82-90.
 50. Ashraf MY, Akhtar K, Sarwar G, Ashraf M. Role of the rooting system in salt tolerance potential of different guar accessions. *Agron Sustain Dev*. 2005;25(2):243-249.
 51. Mubarak AR, Salih NO, Hassabo AA. Fate of 15N-labeled urea under a guar-wheat rotation as influenced by crop residue incorporation in a semi-arid Vertisol. *Trop Agric*. 2015;92(3):172-183.
 52. Satyavathi P, Vanaja M, Reddy A, Vagheera P, Reddy AN, Kumar GV, et al. Identification of suitable guar genotypes for summer season of semi-arid region. *Int J Appl Biol Pharm Technol*. 2014;5(4):71-73.
 53. Choy SY, Prasad KMN, Wu TY, Ramanan RN. A review on common vegetables and legumes as promising plant-based natural coagulants in water clarification. *Int J Environ Sci Technol*. 2015;12(1):367-390.
 54. Rajaprakasam S, Rahman H, Karunakaran S, Babu K, Kulanivelu G, Kambale R, et al. Comparative transcriptome and metabolome profiling in the maturing seeds of contrasting cluster bean (*Cyamopsis tetragonoloba* L. Taub) cultivars identified key molecular variations leading to increased gum accumulation. *Gene*. 2021;791:145727.
 55. Shakir M, Sadaqat HA, Farooq Q, Shabir M, Sodagar A, Nadeem M, et al. A review on guar (*Cyamopsis tetragonoloba* L.): A cash crop. *Int Res J Pharm*. 2020;11(4):1-7.
 56. Gresta F, Ceravolo G, Presti VL, D'Agata A, Rao R, Chiofalo B. Seed yield, galactomannan content and quality traits of different guar (*Cyamopsis tetragonoloba* L.) genotypes. *Ind Crops Prod*. 2017;107:122-129.
 57. Jukanti AK, Bhatt R, Sharma R, Kalia RK. Morphological, agronomic, and yield characterization of cluster bean (*Cyamopsis tetragonoloba* L.) germplasm accessions. *J Crop Sci Biotechnol*. 2015;18(2):83-88.
 58. Mudgil D, Barak S, Khatkar BS. Guar gum: Processing, properties and food application: A review. *J Food Sci Technol*. 2014;51(3):409-418.
 59. Adki KM, Laddha AP, Gaikwad AB, Kulkarni YA. Potential role of seeds from India in diabetes. In: Preedy VR, Watson RR, eds. *InNuts and Seeds in Health and Disease Prevention*. Cambridge, MA: Academic Press; 2020:365-391.

60. Moteriya P, Ram J, Moradiya R, Chanda S. In vitro free radical scavenging and antimicrobial activity of *Cyamopsis tetragonoloba* L. *J Pharmacogn Phytochem*. 2015;4(2).
61. Singh S, Bhagwati D. *Cyamopsis tetragonoloba* (L). Taub.: A Phyto-pharmacological Review. *Human J*. 2016;7(4):166-174.
62. Rasheed MJ, Ahmad K, Ashraf M, Al-Qurainy F, Khan S, Athar HU. Screening of diverse local germplasm of guar [*Cyamopsis tetragonoloba* (L.) Taub.] for salt tolerance: A possible approach to utilize salt-affected soils. *Pak J Bot*. 2015;47(5):1721-1726.
63. Akram M, Zahid M, Farooq ABU, Nafees M, Rasool A. Effects of different levels of NaCl on the seed germination of *Cyamopsis tetragonoloba* L. *Bangladesh J Bot*. 2020;49(3):625-632.
64. Eliasson L. Effects of nutrients and light on growth and root formation in *Pisum sativum* cuttings. *Physiol Plant*. 1978;43(1):13-18.
65. Salama KH, Mansour MM, Hassan NS. Choline priming improves salt tolerance in wheat (*Triticum aestivum* L.). *Aust J Basic Appl Sci*. 2011;5:126-132.
66. Meftahzadeh H, Ghorbanpour M, Asareh MH. Comparison of morphological and phytochemical characteristics in guar (*Cyamopsis tetragonoloba* L.) landraces and cultivars under different sowing dates in an arid environment. *Ind Crops Prod*. 2019;140:111606.
67. Lichtenthaler HK. [34] Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. In: Packer L, Douce R, eds. *Methods in enzymology*. Boca Raton: CRC Press; 1987:350-382.
68. Cornic G. Drought stress and high light effects on leaf photosynthesis. In: Baker NR, Bowyer JR, eds. *Photoinhibition of photosynthesis*. Oxford: Bios Scientific Publishers; 1994:297-313.
69. Velikova V, Yordanov I, Edreva A. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: Protective role of exogenous polyamines. *Plant Sci*. 2000;151(1):59-66.
70. De Vos CHR, Schat H, De Waal MAM, Vooijs R, Ernst WHO. Increased resistance to copper-induced damage of the root cell plasmalemma in copper tolerant *Silene cucubalus*. *Physiol Plant*. 1991;82(4):523-528.
71. Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water-stress studies. *Plant Soil*. 1973;39:205-207.
72. Mukherjee SP, Choudhuri MA. Implications of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in *Vigna* seedlings. *Physiol Plant*. 1983;58(2):166-170.
73. Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem*. 1999;64(4):555-559.
74. Julkunen-Tiitto R. Phenolic constituents in the leaves of northern willows: Methods for the analysis of certain phenolics. *J Agric Food Chem*. 1985;33(2):213-217.
75. Hamilton P, Van Slyke D. Amino acid determination and metal accumulation by *Brassica juncea* L. *Int J Plant Prod*. 1943;3:1735-8043.
76. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72(1-2):248-254.
77. Hodges D, Nozzolillo C. Anthocyanin and anthocyanoplast content of cruciferous seedlings subjected to mineral nutrient deficiencies. *J Plant Physiol*. 1996;147:749-754.
78. Mita S, Murano N, Akaike M, Nakamura K. Mutants of *Arabidopsis thaliana* with pleiotropic effects on the expression of the gene for beta-amylase and on the accumulation of anthocyanin that are inducible by sugars. *Plant J*. 1997;11:841-851.
79. Yemm EW, Willis AJ. The estimation of carbohydrates in plant extracts by anthrone. *Biochem J*. 1954;57(3):508-514.
80. Nelson N. A photometric adaptation of the Somogyi method for the determination of glucose. *J Biol Chem*. 1944;153(2):375-380.
81. Chance B, Maehly AC. [136] Assay of catalases and peroxidases. *Methods Enzymol*. 1955;2:764-775.
82. Gong H, Zhu X, Chen K, Wang S, Zhang C. Silicon alleviates oxidative damage of wheat plants in pots under drought. *Plant Sci*. 2005;169(2):313-321.
83. Sabir P, Ashraf M, Akram NA. Accession variation for salt tolerance in proso millet (*Panicum miliaceum* L.) using leaf proline content and activities of some key antioxidant enzymes. *Journal of Agronomy and Crop Science*. 2011;197(5):340-347.
84. Hafeez MB, Raza A, Zahra N, Shaikat K, Akram MZ, Iqbal S, et al. Gene regulation in halophytes in conferring salt tolerance. *Handbook of Bioremediation*. Cambridge, MA: Academic Press; 2021:341-370.
85. Shabala S, Munns R. Salinity stress: Physiological constraints and adaptive mechanisms. In: Shabala S, eds. *Plant Stress Physiol*. UK: CABI; 2017:24-63.
86. Laohavisit A, Shang Z, Rubio L, Cuin TA, Véry A-A, Wang A, et al. *Arabidopsis* annexin1 mediates the radical-activated plasma membrane Ca²⁺- and K⁺-permeable conductance in root cells. *Plant Cell*. 2012;24(4):1522-1533.
87. Parida AK, Das AB. Salt tolerance and salinity effects on plants: A review. *Ecotoxicol Environ Saf*. 2005;60(3):324-349.
88. Zhao C, Zhang H, Song C, Zhu J-K, Shabala S. Mechanisms of plant responses and adaptation to soil salinity. *The innovation*. 2020;1(1):100017.
89. Dogra V, Li M, Singh S, Li M, Kim C. Oxidative post-translational modification of EXECUTER1 is required for singlet oxygen sensing in plastids. *Nat Commun*. 2019;10(1):2834.
90. Khurana N, Chauhan H, Khurana P. Characterization of a chloroplast localized wheat membrane protein (TaRCI) and its role in heat, drought and salinity stress tolerance in *Arabidopsis thaliana*. *Plant Gene*. 2015;4:45-54.
91. Robinson S, Jones G. Accumulation of glycinebetaine in chloroplasts provides osmotic adjustment during salt stress. *Funct Plant Biol*. 1986;13(5):659-668.
92. Che-Othman MH, Millar AH, Taylor NL. Connecting salt stress signalling pathways with salinity-induced changes in mitochondrial metabolic processes in C 3 plants. *Plant Cell Environ*. 2017;40(12):2875-2905.

93. Tian F, Wang W, Liang C, Wang X, Wang G, Wang W. Overaccumulation of glycine betaine makes the function of the thylakoid membrane better in wheat under salt stress. *Crop J*. 2017;5(1):73-82.
94. Calderon RH, García-Cerdán JG, Malnoë A, Cook R, Russell JJ, Gaw C, et al. A conserved rubredoxin is necessary for photosystem II accumulation in diverse oxygenic photoautotrophs. *J Biol Chem*. 2013;288(37):26688-26696.
95. Salama KH, Mansour MMF. Choline priming-induced plasma membrane lipid alterations contributed to improved wheat salt tolerance. *Acta Physiol Plant*. 2015;37(8):1-7.
96. Sakamoto A, Murata N. The role of glycine betaine in the protection of plants from stress: Clues from transgenic plants. *Plant Cell Environ*. 2002;25(2):163-171.
97. Wei L-X, Lv B-S, Wang M-M, Ma H-Y, Yang H-Y, Liu X-L, et al. Priming effect of abscisic acid on alkaline stress tolerance in rice (*Oryza sativa* L.) seedlings. *Plant Physiol Biochem*. 2015;90:50-57.
98. Sofy MR, Elhawat N, Tarek Alshaal A. Glycine betaine counters salinity stress by maintaining high K^+/Na^+ ratio and antioxidant defense via limiting Na^+ uptake in common bean (*Phaseolus vulgaris* L.). *Ecotoxicol Environ Saf*. 2020;200:110732.
99. Sarker U, Oba S. The response of salinity stress-induced A. tricolor to growth, anatomy, physiology, non-enzymatic and enzymatic antioxidants. *Front Plant Sci*. 2020;11:559876.
100. Odjegba VJ, Chukwunwike IC. Physiological responses of *Amaranthus hybridus* L. under salinity stress. *Indian Journal of Innovations and Developments*. 2012;1(10):742-748.
101. Yildiztugay E, Ozfidan-Konakci C, Kucukoduk M, Duran Y. Variations in osmotic adjustment and water relations of *Sphaerophysa kotschyana*: Glycine betaine, proline and choline accumulation in response to salinity. *Bot Stud*. 2014;55(1):6-9.
102. Athar H-u-R, Zafar ZU, Ashraf M. Glycinebetaine improved photosynthesis in canola under salt stress: Evaluation of chlorophyll fluorescence parameters as potential indicators. *J Agron Crop Sci*. 2015;201(6):428-442.
103. Gong B, Li X, Bloszies S, Wen D, Sun S, Wei M, et al. Sodic alkaline stress mitigation by interaction of nitric oxide and polyamines involves antioxidants and physiological strategies in *Solanum lycopersicum*. *Free Radic Biol Med*. 2014;71:36-48.
104. Santos CV. Regulation of chlorophyll biosynthesis and degradation by salt stress in sunflower leaves. *Sci Hortic*. 2004;103(1):93-99.
105. Cha-um S, Supaibulwatana K, Kirdmanee C. Water relation, photosynthetic ability and growth of Thai Jasmine rice (*Oryza sativa* L. ssp. Indica Cv. KDML 105) to salt stress by application of exogenous glycinebetaine and choline. *J Agron Crop Sci*. 2006;192(1):25-36.
106. Taïbi K, Taïbi F, Ait Abderrahim L, Ennajah A, Belkhodja M, Mulet JM. Effect of salt stress on growth, chlorophyll content, lipid peroxidation and antioxidant defence systems in *Phaseolus vulgaris* L. *S Afr J Bot*. 2016;105:306-312.
107. Minh T, Khang D, Tuyen P, Minh L, Anh L, Quan N, et al. Phenolic compounds and antioxidant activity of Phalaenopsis orchid hybrids. *Antioxidants*. 2016;5(3):31.
108. Moradbeygi H, Jamei R, Heidari R, Darvishzadeh R. Investigating the enzymatic and non-enzymatic antioxidant defense by applying iron oxide nanoparticles in *Dracocephalum moldavica* L. plant under salinity stress. *Sci Hortic*. 2020;272:109537.
109. Wang GP, Li F, Zhang J, Zhao MR, Hui Z, Wang W. Overaccumulation of glycine betaine enhances tolerance of the photosynthetic apparatus to drought and heat stress in wheat. *Photosynthetica*. 2010;48(1):30-41.
110. AbdElgawad H, Zinta G, Hegab MM, Pandey R, Asard H, Abuelsoud W. High salinity induces different oxidative stress and antioxidant responses in maize seedlings organs. *Front Plant Sci*. 2016;7:276.
111. Kovinich N, Kayanja G, Chanoca A, Riedl K, Otegui MS, Grotewold E. Not all anthocyanins are born equal: Distinct patterns induced by stress in Arabidopsis. *Planta*. 2014;240(5):931-940.
112. Nimse SB, Pal D. Free radicals, natural antioxidants, and their reaction mechanisms. *RSC advances*. 2015;5(35):27986-28006.
113. Fiedor J, Burda K. Potential role of carotenoids as antioxidants in human health and disease. *Nutrients*. 2014;6(2):466-488.
114. Akram NA, Shafiq F, Ashraf M. Ascorbic acid-a potential oxidant scavenger and its role in plant development and abiotic stress tolerance. *Front Plant Sci*. 2017;8:613.
115. Naz HIRA, Akram NA, Ashraf M. Impact of ascorbic acid on growth and some physiological attributes of cucumber (*Cucumis sativus*) plants under water-deficit conditions. *Pak J Bot*. 2016;48(3):877-883.
116. Chao WS, Gu Y-Q, Pautot V, Bray EA, Walling LL. Leucine aminopeptidase RNAs, proteins, and activities increase in response to water deficit, salinity, and the wound signals systemin, methyl jasmonate, and abscisic acid. *Plant Physiol*. 1999;120(4):979-992.
117. Qados AMS. Effect of salt stress on plant growth and metabolism of bean plant *Vicia faba* (L.). *J Saudi Soc Agric Sci*. 2011;10(1):7-15.
118. Ashraf MA, Akbar A, Parveen A, Rasheed R, Hussain I, Iqbal M. Phenological application of selenium differentially improves growth, oxidative defense and ion homeostasis in maize under salinity stress. *Plant Physiol Biochem*. 2018;123:268-280.
119. Dhingra HR. Effect of salinity stress on morpho-physiological, biochemical and yield characters of cluster bean [*Cyamopsis tetragonoloba* (L.) Taub.]. *Indian J Plant Physiol*. 2014;19(4):393-398.
120. Ashraf MA, Ashraf M, Shahbaz M. Growth stage-based modulation in antioxidant defense system and proline accumulation in two hexaploid wheat (*Triticum aestivum* L.) cultivars differing in salinity tolerance. *Flora*. 2012;207(5):388-397.
121. Hanin M, Ebel C, Ngom M, Laplaze L, Masmoudi K. New insights on plant salt tolerance mechanisms and their potential use for breeding. *Front Plant Sci*. 2016;7:1787.