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Enhancing canola (*Brassica napus* L.) resilience to salt stress through foliar application of ascorbic acid**

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Abstract. Canola (*Brassica napus* L.), essential for food and biodiesel, suffers from productivity losses due to soil salinity. This study evaluated the effect of ascorbic acid (AsA) foliar spray on mitigating sodium chloride (NaCl)-induced salt stress in two canola varieties, Sandal (V1) and Rachna (V2). Plants were grown under control (0 mM NaCl) and salt stress (150 mM NaCl) conditions, with AsA treatments at 0, 15, and 30 mM concentrations. Salt stress can reduce canola's morphological traits and photosynthetic activity. However, AsA treatment increased total chlorophyll, chlorophyll *a*, chlorophyll *b* (each by 55%), and carotenoids (62.8%). Salt stress elevated reactive oxygen species (ROS) and Na^+ accumulation, but AsA application under stress enhanced glycine betaine (24%), proline (23.8%), anthocyanin (26%), total soluble sugars (24.6%), proteins (19.8%), endogenous AsA (26%), flavonoids (19.8%), total phenolics (29%), and antioxidant enzymes (SOD by 24%, POD by 19.7%, and CAT by 20%). The AsA treatments helped to control oxidative stress, regulate ion balance, and reduce lipid peroxidation, thereby preventing ROS overproduction. Out of two varieties, Rachna (V2) performed better than Sandal (V1) across all morphological and physiological parameters, demonstrating superior resilience under salinity.

Keywords: canola, salinity, ascorbic acid, antioxidants, ions

1. INTRODUCTION

Numerous biotic and abiotic factors exert substantial influence on vegetation growth, crop production and the quality of food produced (Ilyas *et al.,* 2024). Salt stress triggers various physiological, morphological and molecular alterations in plants, impeding their growth and developmental abilities and leading to approximately 50% reduction in crop yield (Sachdev *et al.,* 2021; Zafar *et al.,* 2024). Global agricultural productivity in field crops is impacted by salinity, posing a serious danger to countries whose economies are dependent on agriculture (Hualpa-Ramirez *et al.,* 2024). Water logging, inadequate irrigation techniques, high fertilizer usage in agriculture and intrusion of seawater are among the primary causes of salinity in Pakistan (Chaudhary *et al.,* 2024). Out of all abiotic stresses, salinity stress alone impacts over 33% of irrigated land and approximately 20% of the total global cultivated area (Mukhopadhyay *et al.,* 2021). Growth inhibition is one of the main effects of salinity stress on plants (Omara *et al.,* 2022). High salt concentrations in the soil prevent roots from absorbing water, resulting in water shortages in plant tissues. Lack of water and osmotic stress from too much

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salt prevent plants from growing (Naz *et al.,* 2022). Plants may consequently show decreased biomass accumulation, slowed growth and decreased shoot and root development (Kumar *et al.,* 2024).

Salinity induces osmotic stress, typically marked by the presence of sodium ions (Na^+) and chloride ions (Cl^-) in the soil. This elevation in salt concentration in the soil solution reduces plant water absorption (Maqsood *et al.,* 2021). Excessive salinity from the external environment causes ion toxicity, nutritional imbalances (Farhat *et al.,* 2023), reactive oxygen species (ROS) production, cell membrane leakage and metabolic distortions that inhibit growth and yield (Zoufan *et al.,* 2020). Higher levels of malondialdehyde (MDA) indicate that salinity increases the degree of lipid peroxidation, which leads to increased membrane damage as a result of unbalanced redox management (Khalid *et al.,* 2024). Because of this, a lot of plants develop tolerance to salt in their cells and most of them build defence mechanisms against it. Various ions, such as Na^+, Cl^-, Ca^{2+}, Mg^{2+}, SO_4^{2-}, NO^{3-}, HCO_3 and K^+, are associated with soil salinity and adversely affect crucial metabolic processes (Ahmed *et al.,* 2019). Therefore, regular growth and development in saline environments could be achieved by optimizing photosynthetic systems, such as electron transport and chlorophyll pigment systems (Hasanuzzaman *et al.,* 2023), raising levels of antioxidant compounds, such as ascorbate (ASA), glutathione (GSH), salicylate and α-tocopherol, and increasing the activity of plant antioxidant enzymes, such as superoxide dismutase, peroxidase dismutase, catalase and ascorbate peroxidase (Kanwal *et al.,* 2024).

Canola (*Brassica napus* L.), commonly known as rapeseed, is a significant and extensively cultivated crop globally in the Brassicaceae family (Dawood *et al.,* 2023). It is the second-most popular oilseed crop (after soybean) in terms of seed cake used for animal feed and edible oil for humans (Zhang *et al.,* 2024). It contributes significantly to the world's edible vegetable oil supply, making up about 20% of the total oil produced worldwide (Siles *et al.*, 2021). Furthermore, rapeseed is well-known for its rich content of fatty acids, especially vitamins and essential fatty acids (Shen *et al.,* 2023). This plant offers beneficial agricultural and nutritive qualities, including low cholesterol and resilience to cold, salinity and drought stressors (Wang *et al.,* 2023). Secondary metabolites found in Brassica oilseeds and other crops have received much-needed attention as a result of their agronomic, biological and economic benefits (Elsawy *et al.,* 2022).

During its growth, oilseed rape is subjected to a variety of abiotic challenges, including salt stress (Raza, 2021). The suppression of biochemical, physiological, and metabolic systems resulting from osmotic stress, ion toxicity, and reduced water and mineral intake is the primary cause of the deleterious effects of salt stress on canola growth and development (Rezayian and Zarinkamar, 2023). Salinity stress reduces rapeseed plants' height, leaf area and photosynthesis, shoot growth and eventually yield (Shahzad *et al.,* 2022). Plants can lessen the harm caused by salt stress by means of physiological, biochemical, and molecular regulatory processes, such as signaling, the activation of antioxidant enzymes, controlling osmotic pressure and ion homeostasis, and scavenging reactive oxygen species (Wang *et al.,* 2022). Osmolytes serve as immediate sources of energy during stress recovery, stabilize membranes and macromolecules, act as antioxidants, buffer the cellular redox potential, and preserve the functional balance of the cell, all of which safeguard plant cells (Rasheed *et al.,* 2014). Additionally, it is hypothesized that the safeguarding of cellular membranes, crucial for salt tolerance, is attained through the protection of enzymes such as superoxide dismutase, peroxidase, and catalase, guarding against excessive ROS production induced by salt and membrane lipid peroxidation (Mohamed *et al.,* 2020).

Ascorbic acid (AsA), commonly referred to as vitamin C, is a crucial non-enzymatic antioxidant that helps plants maintain normal function and cope with stress, including salt stress, even in challenging conditions (El-Beltagi *et al.,* 2020). Plant cells naturally produce ascorbic acid, of which 30-40% is found in fully developed chloroplasts. Because of the close relationship between ascorbic acid and chloroplasts, exogenous application of ascorbic acid to stressed plants resulted in increased pigment production during photosynthesis (Xu and Huang, 2018). It plays an essential role in the metabolism of reactive oxygen species (ROS) by acting as a crucial "redox buffer" (Billah *et al.,* 2017). Ascorbic acid (AsA) supplementation has been demonstrated to enhance ascorbate peroxidase (APX) activity in stressed plants and efficiently detoxify hydrogen peroxide (H_2O_2) at the cellular level (Wu *et al.,* 2024). The endogenous levels of ascorbic acid decreased by exposure to certain abiotic stressors, on the other hand, these levels can be restored by the exogenous application of ascorbic acid (Wang *et al.,* 2019). The application of ascorbic acid improves cell division and elongation by promoting the expansion of the cell wall and retarding the stiffening of the cell wall, hence lowering lignification in meristematic cells (Ortiz-Espín *et al.,* 2017).

Since antioxidants are essential for mitigating the negative effects of environmental stressors on plants, either directly or indirectly. Considering the importance of the canola crop and ascorbic acid (AsA), this study was carried out to determine whether foliar application of ascorbic acid (AsA) can reduce the negative impacts of salt stress on canola plants.

2. MATERIALS AND METHODS

An experiment was conducted at the Botanical Garden, The Islamia University of Bahawalpur, to study the impact of salt stress on different canola varieties using foliar

application of ascorbic acid (AsA). The study was carried out by using a completely randomized design (CRD) with three replicates. Seed of two canola varieties, Sandal and Rachna was acquired from Ayub Agricultural Research Institute, Faisalabad and were exposed to two salinity levels (Control and 150 mM NaCl). Foliar applications of three ascorbic acid concentrations (0, 15, and 30 mM) were applied. For planting, plastic pots were filled with 8 kg of soil along with sowing of fifteen seeds in each pot. After two weeks of germination, seven plants were retained after thinning. After 45 days of seed germination, salt stress was applied in successive intervals following the method of Islam *et al*. (2016). The initial ECe of soil was 9 mM. The salinity was maintained in successive time intervals to avoid the sudden salt injury. In first watering, 50 mM salinity was established using NaCl and measured through ECe meter (STARTER 3100). After two days of first salinity application, 100 mM salinity was sustained through second watering by adding NaCl. Third watering was applied to maintain the final 150 mM salinity. On 57-day-old plants, foliar fertigation of AsA at three different concentrations (0, 15 and 30 mM) was carried out. To prevent leaf sunburn, a 15 mL solution of each AsA concentration was given to each pot just before dusk. Tween 20 (0.1%) was utilized as the surfactant to ensure maximum absorption. Following three weeks of foliar spraying on each replicate, the plants were carefully pulled out, cleaned, and data was noted for different morphological parameters. For biochemical parameters, fourth leave from the top of the plant was collected for chlorophyll pigments, biochemical attributes and antioxidant enzymes.

2.1. Morphological traits

After carefully removing one plant from each pot, the lengths of the shoot and root were measured with a measuring tape. With an electrical balance, the fresh weights of the root and shoot were determined. Plant shoot and root dry weights were determined by carefully drying the samples in an oven at 65°C.

2.2. Photosynthetic pigments

Using Arnon's (1949) approach, the levels of carotenoids, chlorophyll *a*, chlorophyll *b*, and total chlorophyll were determined. 0.1 g of the leaves were crushed up in 80% acetone. The sample was stored for the night. The reading was taken the following day at 480, 645 and 663 nm using a spectrophotometer.

2.3. Reactive oxygen species

2.3.1. Hydrogen peroxide (H_2O_2)

Following Velikova *et al*. (2000), hydrogen peroxide (H_2O_2) levels were determined using a modified protocol. A 0.25 g leaf sample was homogenized in 2 mL of ice-cold 0.1% TCA. The homogenate was centrifuged at 1500 rpm for 20 min to collect the supernatant. Afterwards, 1 mL of potassium iodide (KI) solution, 0.5 mL of phosphate buffer, and 0.5 mL of the supernatant were added to a test tube and it was gently vortexed and the absorbance was measured at 390 nm using a spectrophotometer.

2.3.2. Malondialdehyde (MDA)

Yagi (1982) method was used to measure malondialdehyde (MDA) content. A 0.25 g fresh leaf sample was crushed in 2 mm of 0.1% TCA, centrifuged at 1 500 rpm for 20 min, and the supernatant collected. The supernatant (1 mL) and TCA-TBA solution (4 mL) were incubated at 95°C for 30 min, cooled, and absorbance measured at 532 and 600 nm.

2.4. Enzymatic-antioxidants

Fresh leaf material of 0.25 g was homogenized by using 5 mL potassium phosphate buffer (pH 7.8) in pestle and mortar at 4 °C. The homogenized extract was centrifuged for 15 min at 12 000 rpm. The extracted material was stored at -20°C to find superoxide dismutase, catalase and peroxidase activities.

2.4.1. Superoxide dismutase (SOD)

Giannopolitis and Ries (1977) technique was used to assess superoxide dismutase (SOD) activity. The reaction mixture was composed of 50 µL of nitroblue tetrazolium (NBT), 50 µL of riboflavin, 100 µL of L-methionine, 250 µL of phosphate buffer, 100 µL of Triton-X, 150 µL of distilled water, and 50 µL of the sample. The mixture was placed under light for 20 min and absorbance was recorded at 560 nm by using spectrophotometer.

2.4.2. Catalase (CAT)

Chance and Maehly (1955) protocol was used to estimate the activity of catalase. The reaction mixture was prepared by using 1 ml distilled water, 0.1 ml sample extract and 1.9 ml potassium phosphate buffer. Spectrophotometer was used to check decrease in absorbance for 2 min at 240 nm after 30 s interval.

2.4.3. Peroxidase (POD)

To determine POD activity through Chance and Maehly (1955) method, put 7.5 mL of phosphate buffer, 0.1 mL of guaicol solution (335 µL H_2O_2+ 15 mL phosphate buffer), 0.1 mL of H_2O_2 solution (100 mL H_2O_2+20 mL phosphate buffer), and 0.05 mL of sample extract in a cuvette. Increase in absorbance was measured at 470 nm, by 30 s difference till 2 min with the help of spectrophotometer.

2.5. Ascorbic acid

Ascorbic acid were measured using the method by Mukherjee and Choudhuri (1983). A 0.5 g leaf sample was ground in 10 mL of 6% (w/v) trichloroacetic acid. After filtration, 2% diphenylhydrazine, thiourea, and 80% H_2SO_4

were added to the filtrate. The optical density (OD) of the treated samples was then read at 530 nm to determine the AsA content.

2.6. Total soluble sugars (TSS)

The method developed by Yemm and Wills (1954) was used to calculate total soluble sugars. 0.5 g of leaves were boiled in 5 ml of distilled water for an hour by using test tube. The extract was filtered and water was added to make the volume 25 ml. Anthrone reagent (2.5 mL) and 0.5 mL of leaf extract were mixed in a test tube and kept in water bath for 20-30 min. After cooling, absorbance was recorded at 620 nm by spectrophotometer.

2.7. Total soluble proteins (TSP)

Bradford (1976) method was used to estimate the total soluble proteins. 5 ml potassium phosphate buffer (pH 7.8) was used to crush 0.25 g dry leaf material in pestle and mortar. The extract was centrifuged for 10 minutes at 12 000 rpm at 4ºC. 100 µl of extract and 5 ml Bradford reagent was mixed in test tube and vortexed for 10 s. Absorbance was noted at 595 nm by using spectrophotometer.

2.8. Glycine betaine (GB)

To record the glycine betaine concentration, Grieve and Grattan (1983) method was used. Fresh leaf material (0.25 g) was extracted in 5 ml of distilled water. At 12 000 rpm, the extract was centrifuged for 15 min. After mixing 2 ml of NH_2SO_4 with 1 ml of the sample, 500 µl of this mixture was added to a test tube. Test tubes were cooled for 90 min following the addition of 0.2 ml of potassium tri-iodide. After that, test tubes were chilled on ice, add 2.8 ml of distilled water and 6 ml of 1, 2-dichloroethane. Two different layers were formed in a test tube, and the absorbance of lower layer was measured at 365 nm by using a spectrophotometer.

2.9. Total free proline

To determine total free proline contents by Bates *et al*. (1973) method, fresh leaf material (0.25 g) was crushed and filtered after being dissolved in 5 mL of 3% sulfosalicylic acid. 1 mL of filtrate, 1 mL of acid ninhydrin, and 1 mL of glacial acetic acid were mixed in a test tube and heated in a water bath for 90 min at 100°C. After that, 2 mL of toluene was added and samples were placed in ice to cool them. Two layers formed after the vortexing, and the spectrophotometer was used to measure the absorbance of the top pinkish layer at 520 nm.

2.10. Total phenolics

According to Julkenen-Titto (1985), the amount of total phenolic was recorded. A 10 mL solution of 80% acetone was used to extract the fresh leaf material of 0.5 g. The supernatant (1 mL) was mixed with 5 mL of Na_2CO_3 (20%) and 1 mL of Folin-Ciocalteu phenol reagent. To make the mixture 10 mL in volume, distilled water was utilized. The absorbance of the reaction mixture was measured at 750 nm with the help of spectrophotometer.

2.11. Anthocyanin

Anthocyanin contents were recorded according to Murray and Hackett (1991). In 5 ml of acidified methanol, 0.2 g of the leaf sample was crushed. By dissolving 1 mL of HCl in 120 mL of methanol, acidified methanol was prepared. After labelling the test tube carefully, transfer the sample into it. Then, test tube was placed in water bath at 50°C for 60 min. The spectrophotometer was used to record absorbance at 535 nm.

2.12. Flavonoids

To measure flavonoids by using Marinova *et al*. (2005) method, 300 µL of $NaNO_3$ and 1 ml of the ethanol extract was added in a test tube, followed by a short incubation at 25°C. Following the addition of 300 µL of $AlCl_3$, for 5 min, the mixture was maintained at room temperature. The mixture has been left to cool at room temperature for 10 min after adding 2 ml of NaOH. The mixture was diluted to a level of 10 mL using distilled water. At 510 nm, the absorbance was measured with the help of spectrophotometer.

2.13. Ion analysis (Na^+, K^+, and Ca^{2+})

Oven-dried shoot and root samples (0.1 g) were combined with 5 mL of H_2SO_4, left overnight, and heated at 100°C with H_2O_2. After cooling, the mixture was filtered and diluted to 50 mL. Na^+, K^+ and Ca^{2+} concentrations in roots and shoots were measured using a flame photometer, method given by Wolf *et al*. (1982).

2.14. Statistical analysis

A three-way (2 × 2 × 3) full factorial design was applied with three replications (Table 1). Statistix 8.1 was used to conduct three way analysis of variance (ANOVA) to estimate the main effects and various interactions among AsA concentration, varieties and NaCl levels. By using significance level at $p < 0.05$, Tukey's HSD was performed for mean comparison. To visualize trends and interrelationships among variables, Pearson's correlation coefficients were calculated using the corrplot package (v0.94) in R software (v4.3.2, R Core Team 2023). Multivariate analysis was further performed through Principal Component Analysis (PCA) using the FactoMineR (v2.9) and factoextra (v1.0.7) packages in R to reduce dimensionality and explore group clustering among treatments. Graphical presentations of the results were also generated using R software (v4.3.2, R Core Team 2023).

Table 1. Three-way (2x2x3) full factorial analysis of variance showing proportion of variance (effect size) of each source of variation (SOV) in 29 studied parameters

DV	Variety (V)	Stress (S)	AsA (A)	V x S	V x A	S x A	VxSxA	Error
df	1	1	2	1	2	2	4	24
R_Na	0.027***	0.822***	0.094***	0.003	0.001	0.012*	0.000	0.041
S_Na	0.028***	0.835***	0.092***	0.003	0.000	0.008.	0.000	0.033
R_K	0.017***	0.823***	0.132***	0.001	0.000	0.002	0.000	0.025
S_K	0.013***	0.888***	0.082***	0.000	0.000	0.002	0.000	0.013
R_Ca	0.017**	0.824***	0.121***	0.000	0.001	0.007.	0.000	0.031
S_Ca	0.016***	0.890***	0.069***	0.000	0.000	0.002	0.000	0.022
Proline	0.030**	0.702***	0.179***	0.007	0.000	0.006	0.003	0.072
GB	0.046***	0.696***	0.210***	0.001	0.000	0.001	0.000	0.046
Flavonoids	0.014*	0.712***	0.222***	0.001	0.001	0.000	0.001	0.049
Phenolics	0.017**	0.773***	0.151***	0.000	0.000	0.004	0.000	0.053
TSS	0.044***	0.742***	0.181***	0.002	0.001	0.000	0.000	0.030
AA	0.016*	0.698***	0.229***	0.001	0.000	0.000	0.000	0.056
Anthocyanin	0.020**	0.717***	0.205***	0.001	0.000	0.000	0.001	0.056
SOD	0.026***	0.747***	0.180***	0.002	0.000	0.000	0.003	0.041
POD	0.044***	0.768***	0.155***	0.004.	0.000	0.001	0.000	0.028
CAT	0.025**	0.766***	0.160***	0.003	0.001	0.000	0.000	0.045
MDA	0.005.	0.833***	0.125***	0.000	0.000	0.001	0.001	0.036
H_2O_2	0.013***	0.841***	0.120***	0.000	0.000	0.001	0.001	0.023
Chl_a	0.006**	0.933***	0.045***	0.001	0.000	0.000	0.000	0.014
Chl_b	0.009***	0.892***	0.071***	0.001	0.001	0.013***	0.000	0.013
T_Chl	0.007***	0.925***	0.054***	0.001.	0.000	0.002.	0.000	0.009
Carotenoids	0.010***	0.912***	0.064***	0.001	0.000	0.001	0.000	0.012
RFW	0.038***	0.851***	0.088***	0.001	0.001	0.002	0.001	0.018
RDW	0.009*	0.839***	0.116***	0.001	0.002	0.004	0.001	0.028
SFW	0.054***	0.729***	0.182***	0.000	0.002	0.000	0.000	0.032
SDW	0.038***	0.812***	0.118***	0.001	0.000	0.003	0.000	0.028
RL	0.017*	0.583***	0.298***	0.000	0.005	0.013	0.005	0.079
SL	0.053***	0.806***	0.119***	0.002	0.001	0.002	0.000	0.018
TSP	0.181***	0.609***	0.163***	0.008*	0.002	0.001	0.001	0.036

*** $p < 0.001$ highly significant, ** $p < 0.01$ very significant, * $p < 0.05$ significant. Df – degrees of freedom.

3. RESULTS

3.1. Morphological parameters

In both varieties, the application of 150 mM NaCl showed a significant reduction in root and shoot lengths, as well as fresh and dry weights (Fig. 1). Root length, root fresh weight and root dry weight exhibited a notable reduction of 29.7, 60 and 52.4% in Sandal, and 29.4, 52.7 and 48.6% in Rachna, respectively. Exogenous application of 30 mM AsA under salt stress improved root length, root fresh and dry weights in Sandal by 42.7, 42.9 and 47, and 39.2, 50.4 and 38.7%, respectively, in Rachna. The impact of salinity on Sandal resulted in a decrease of 42.69% in shoot length, 42.97% in shoot fresh weight and 57% in shoot dry weight. Similarly, in Rachna there was a reduction of 36% in shoot length, 36.41% in shoot fresh weight and 54.7% in shoot dry weight. However, foliar spray of ascorbic acid increased shoot length, shoot fresh weight and dry weight by 27.98, 41.4 and 44.7% in Sandal, and 29.76, 38.9, and 57% in Rachna.

3.2. Photosynthetic pigments and reactive oxygen species (ROS)

The findings indicated a significant reduction in levels of chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids when both canola varieties were exposed to 150 mM salt stress (Fig. 2). In response to salinity, the concentrations

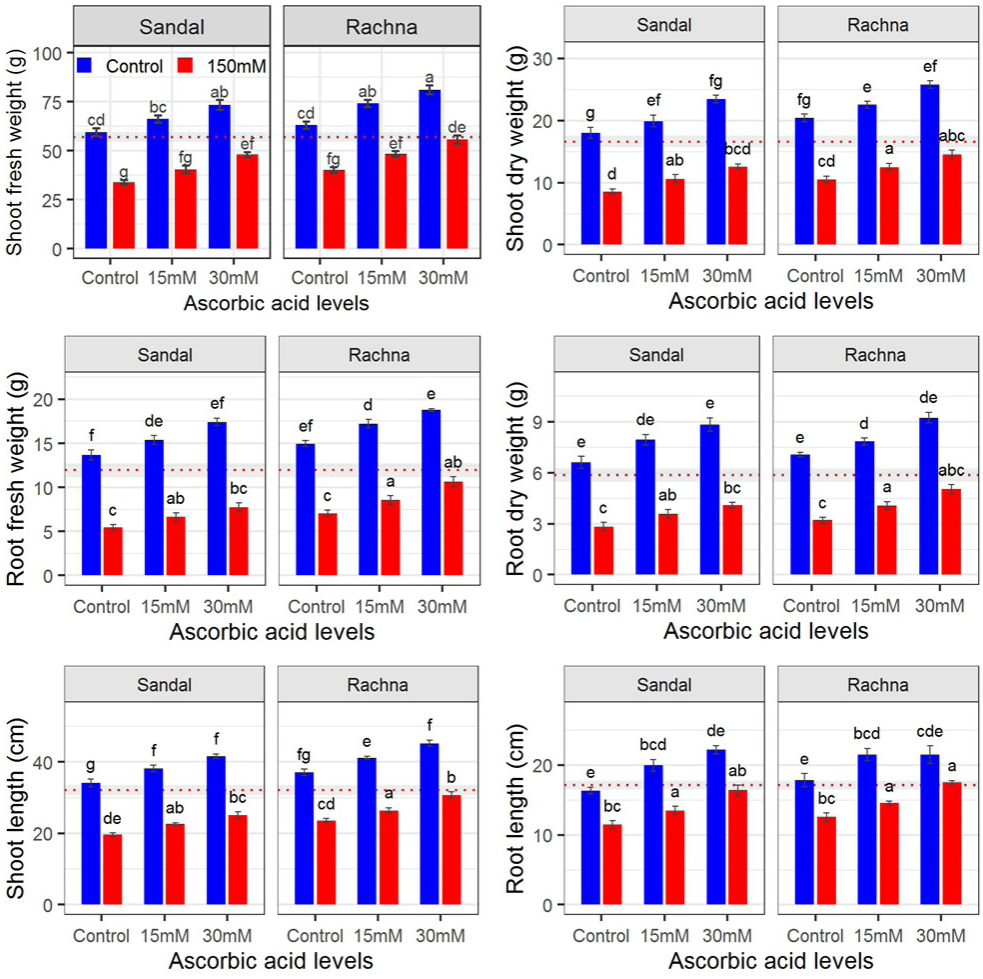

Fig. 1. Impact of exogenously applied ascorbic acid (AsA) on various morphological parameters of canola under saline conditions. Error bars represent the standard error (SE) across three replicates. For each parameter, means labeled with identical letters are not significantly different ($p < 0.05$). Blue bar – control (0 mM NaCl), red bar – salinity (150 mM NaCl). The dotted horizontal line indicates the overall mean, and the grey rectangle along the dotted line shows the standard error of that overall mean.

of chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids decreased by 70, 72.6, 70.9 and 69% in Sandal, and 65.9, 65.8, 65.8 and 65% in Rachna, respectively. However, application of 30 mM AsA under salt stress led to enhancements in the concentrations of chlorophyll *a* and *b*, total chlorophyll, and carotenoids in Sandal, reaching levels of 53.9, 46.5, 51.7, and 58.5%, and in Rachna, with values of 55, 55, 55 and 62.8%, respectively. On the contrary, 150 mM NaCl application increased oxidative damage, as evidenced by increasing levels of reactive oxygen species (H_2O_2 and MDA) in both varieties (Fig. 2). In response to 150 mM NaCl stress, the concentrations of H_2O_2 and MDA increased significantly by 60 and 67.7% in Sandal, and 62 and 73.5% in Rachna, respectively. However, foliar application of 30 mM ascorbic acid under salt stress alleviated the negative effects of salinity, resulting in a decrease in H_2O_2 and MDA levels of 18 and 20% in Sandal, and 18 and 20.5% in Rachna, accordingly.

3.3. Enzymatic antioxidants (SOD, POD, CAT), total soluble proteins, total soluble sugars and flavonoids

The activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) considerably increased in both canola varieties (Fig. 3). Under salt stress, Sandal exhibited a notable increase of 53, 48.8 and 57% in superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities, while Rachna experienced corresponding increases of 61, 51 and 60%, without the AsA application. The application of 30 mM ascorbic acid under salt stress led to a substantial increase in the activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) by 24, 19.7 and 19% in Sandal, and 17, 19 and 20% in Rachna, correspondingly. Similarly, under saline conditions, both canola varieties showed a significant increase in total soluble protein (TSP), total soluble sugars (TSS) and flavonoids with AsA foliar application (Fig. 3). In response to salt stress, Sandal showed considerable increases of 58.8% in total soluble protein (TSP), 65.7% in total soluble

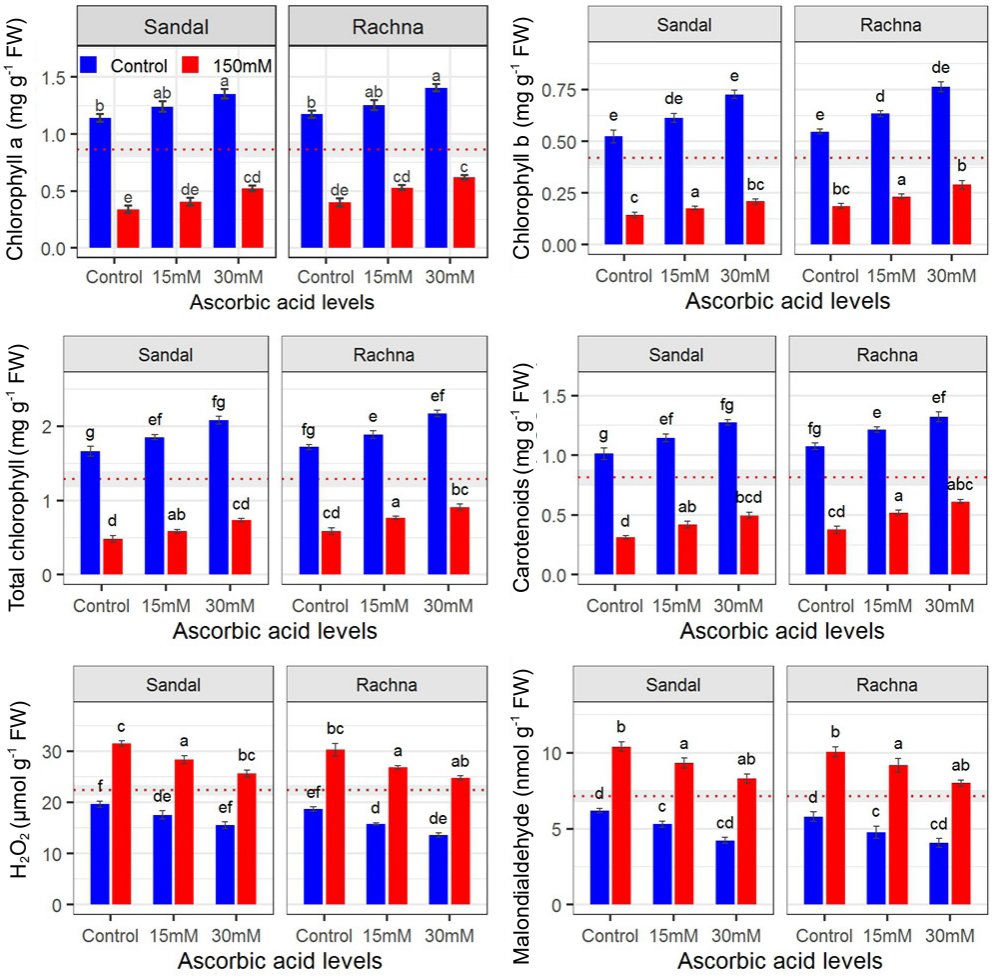

Fig. 2. Impact of exogenously applied ascorbic acid (AsA) on photosynthetic pigments, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) of canola under saline conditions. Error bars represent the standard error (SE) across three replicates. For each parameter, means labeled with identical letters are not significantly different ($p < 0.05$). Blue bar – control (0 mM NaCl), red bar – salinity (150 mM NaCl). Other explanations as in Fig. 1.

sugars (TSS) and 44% in flavonoids. Meanwhile, Rachna showed increases of 67% in total soluble protein (TSP), 65.9% in total soluble sugars (TSS) and 40.5% in flavonoids. The foliar application of 30 mM ascorbic acid under saline environment, resulted in an improvement of 19.8% in total soluble protein (TSP), 24% in total soluble sugars (TSS) and 19.8% in flavonoids in Sandal, and 19, 24.6 and 19% in Rachna, respectively.

3.4. Anthocyanin, total free proline, glycine betaine (GB), phenolics and ascorbic acid

After being exposed to 150 mM salt stress, the levels of anthocyanin, total free proline and glycine betaine increased in both canola varieties (Fig. 4). According to statistical data, under saline environment, Sandal showed an increase in the amounts of anthocyanin, total free proline and glycine betaine by up to 58, 42 and 47%, accordingly, while Rachna increased the amounts by up to 57, 39 and 46%, respectively. Applying 30 mM ascorbic acid resulted in a considerable rise in anthocyanin, total free proline and glycine betaine contents in Sandal (26, 21.8, and 24%) and Rachna (22.6, 23.8, and 20.8%) under saline conditions, when 30 mM AsA was applied. Similarly, under saline conditions, Sandal exhibited increases in phenolics (75%) and ascorbic acid (58%), while Rachna showed increases in phenolics (67.7%) and ascorbic acid (57%) (Fig. 4). However, under salt stress along with 30 mM AsA application, the application of ascorbic acid led to a significant increase in both phenolics and ascorbic acid contents, reaching up to 27.8 and 26% in Sandal and 29 and 22.6% in Rachna, correspondingly.

3.5. Root and shoot ions (Na^+, K^+ and Ca^{2+})

Application of salt stress (150 mM NaCl) resulted in a notable increase in detrimental Na^+ ions in both the root and shoot of both canola varieties (Fig. 5). The levels of Na^+ in the both root and shoot increased significantly in Sandal (75 and 56.9%) and Rachna (73 and 52.8%), respectively.

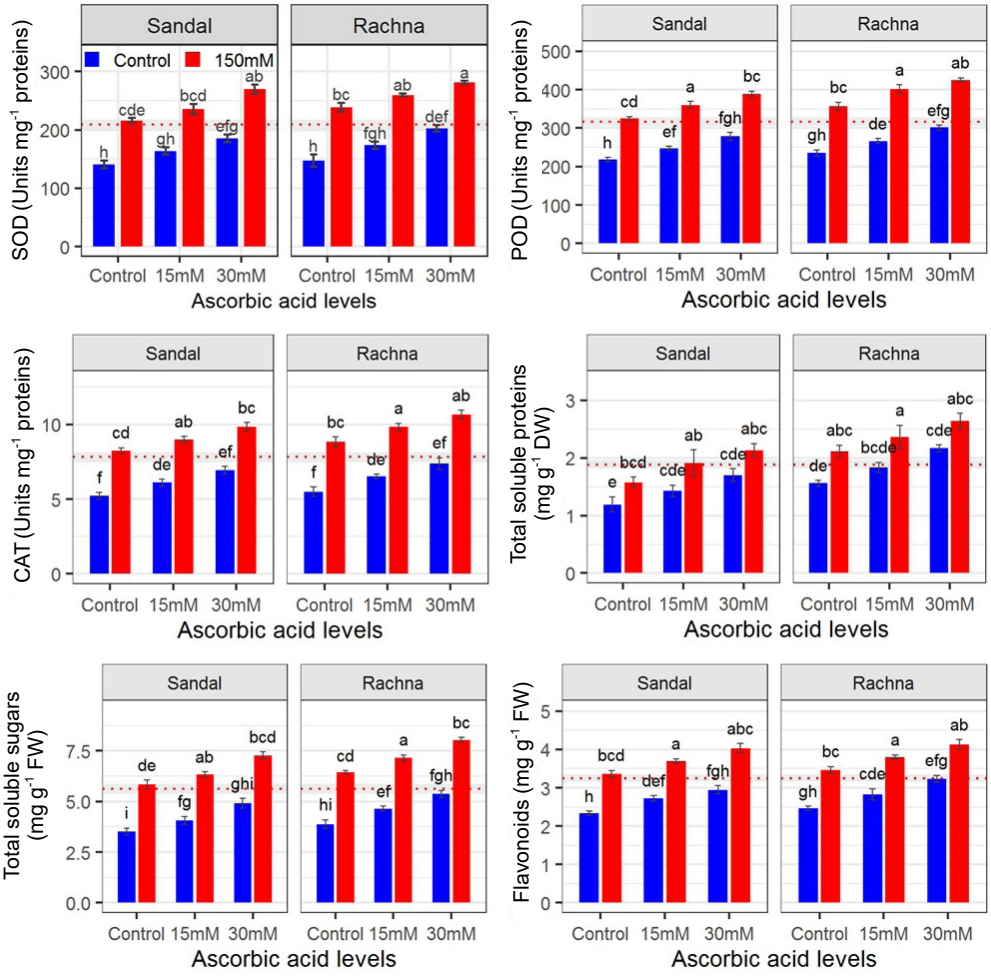

Fig. 3. Impact of exogenously applied ascorbic acid (AsA) on superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), total soluble proteins, total soluble sugars and flavonoids of canola under saline conditions. Error bars represent the standard error (SE) across three replicates. For each parameter, means labeled with identical letters are not significantly different ($p<0.05$). Blue bar – control (0 mM NaCl), red bar – salinity (150 mM NaCl). Other explanations as in Fig. 1.

However, foliar spray of 30 mM AsA under salt stress, decreased the contents of root and shoot Na^+ up to 19.6 and 18% in Sandal, and 20.6 and 16.5%, respectively, in Rachna. Salinity significantly decreased the beneficial root and shoot K^+, as well as root and shoot calcium Ca^{2+}, in Sandal (53, 59, 52, and 53.9%) and Rachna (48.6, 55.5, 47.7, and 50.7%), accordingly (Fig. 5). Contrastingly, foliar application of 30 mM ascorbic acid under saline environment, resulted in significant improvements in both root and shoot potassium (K^+) by up to 35 and 66.6%, and root and shoot calcium (Ca^{2+}) by up to 34.6 and 41% in Sandal and 33.9, 59, 26, and 45% in Rachna, respectively.

3.6. Pearson's correlation coefficient (r) of 29 parameters

Pearson's correlation coefficients (r) were calculated for 29 variables, each with 12 observations. The variables were ordered based on their first principal component (FPC) scores. Variables with the highest importance, exhibiting positive correlations with each other and with the FPC, are highlighted in blue (Fig. 6). A correlation coefficient exceeding 0.823 is considered statistically significant ($p<0.001$). All parameters in the correlogram, from MDA (at the top) to TSP (below), show significant positive correlations with each other ($p<0.001$). Conversely, parameters from RL to chlorophyll-a (chl_a) demonstrate negative correlations within this group.

The correlogram is visually divided into two distinct blocks: a blue block above representing positive correlations and a brownish-red block below illustrating negative correlations among the parameters (Fig. 6). Furthermore, potassium and calcium levels in both roots and shoots exhibit an inverse relationship with the parameters spanning from MDA to TSP (top left to bottom) within the correlation matrix. Parameters like MDA, H_2O_2, and shoot/root sodium exhibit positive correlations with significant ($p<0.001$). However, these parameters are also significantly ($p<0.001$) negatively correlated with root length to chlorophyll-a.

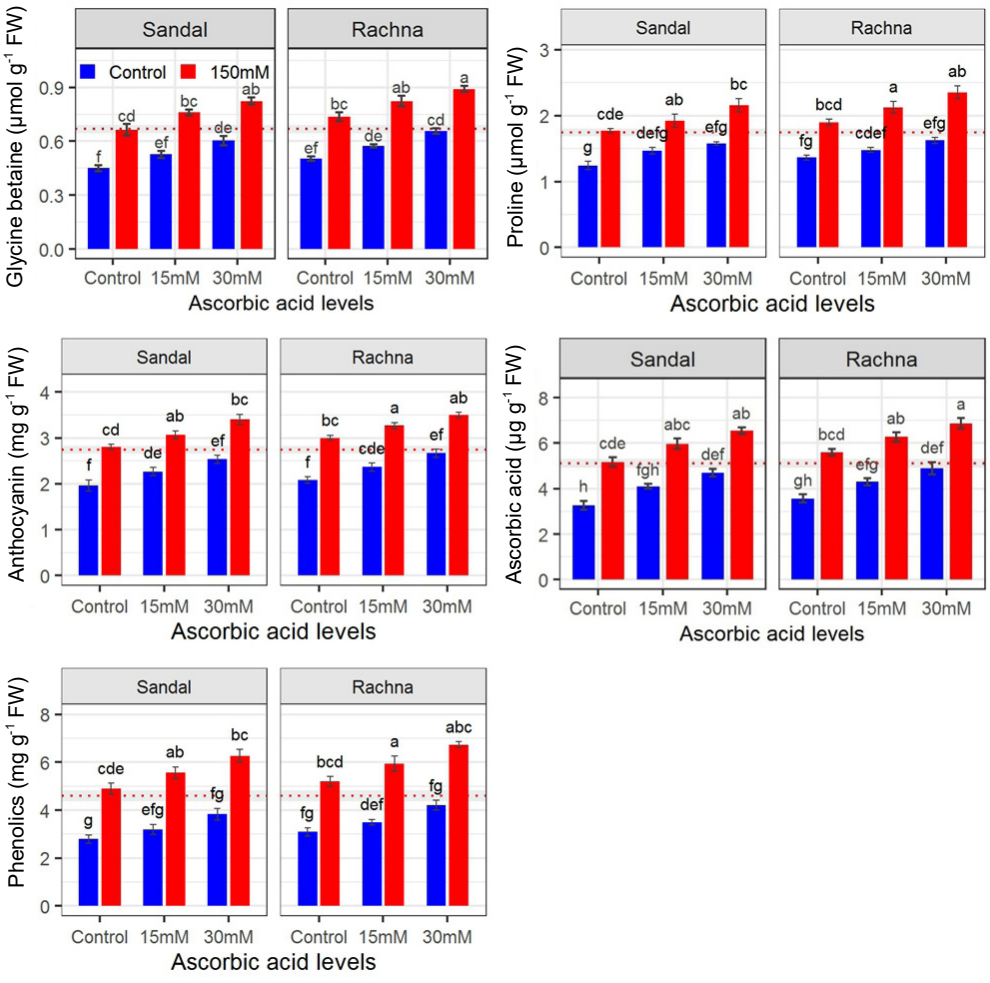

Fig. 4. Impact of exogenously applied ascorbic acid (AsA) on glycine betaine, proline, anthocyanin, ascorbic acid and total phenolics of canola under saline conditions. Error bars represent the standard error (SE) across three replicates. For each parameter, means labeled with identical letters are not significantly different ($p < 0.05$). Blue bar – control (0 mM NaCl), red bar – salinity (150 mM NaCl). Other explanations as in Fig. 1.

3.7. Principal component analysis (PCA)

A dataset with 29 parameters (columns) and 12 treatments (rows) was analyzed using principal component analysis (PCA). This revealed two important components (PCs) shown in Fig. 7a. First principal component (PC1) explained most of the variation (82%) with an eigenvalue of 23.85. It captured factors related to chlorophyll and shoot length, which clustered together on the x-axis in Fig. 7b due to their similar patterns. The second principal component (PC2) explained the remaining variation (17%) with an eigenvalue of 4.9. It captured factors related to TSP and SFW, which clustered together on the y-axis in Fig. 7c.

In essence, PCA separated the variables into two groups, group-1 (16 variables), clustered around PC1, likely representing factors influencing chlorophyll and shoot length. While group-2 (13 variables), clustered around PC2, likely representing factors influencing TSP and SFW. Figure 2d further clarifies two varieties v1 and v2 under control (s1) and stressed (s2) conditions, with varying ascorbic acid (AsA) levels, are shown. Their 12 treatment observations are spread across PC2, with a clear separation between s1 and s2. All 29 parameters are positioned based on PC1 and PC2, forming two distinct groups. Parameters on the left side of the figure 7d (biplot) are associated with higher values of v1 and v2 under normal conditions (s1). Parameters on the right side are associated with higher values under stress conditions (s2).

High cumulative variance explained by first two PCs validates the grouping of parameters. PC1 captured variation in chlorophyll content and shoot length, which are key indicators of photosynthetic efficiency and vegetative growth. Clustering of these traits suggested a shared physiological response to treatment, particularly under normal (S1) conditions. PC2 highlighted variation in total soluble proteins (TSP) and shoot fresh weight (SFW), which are critical markers of osmotic adjustment and biomass accumulation under stress (S2) conditions. The biplot (Fig. 2d)

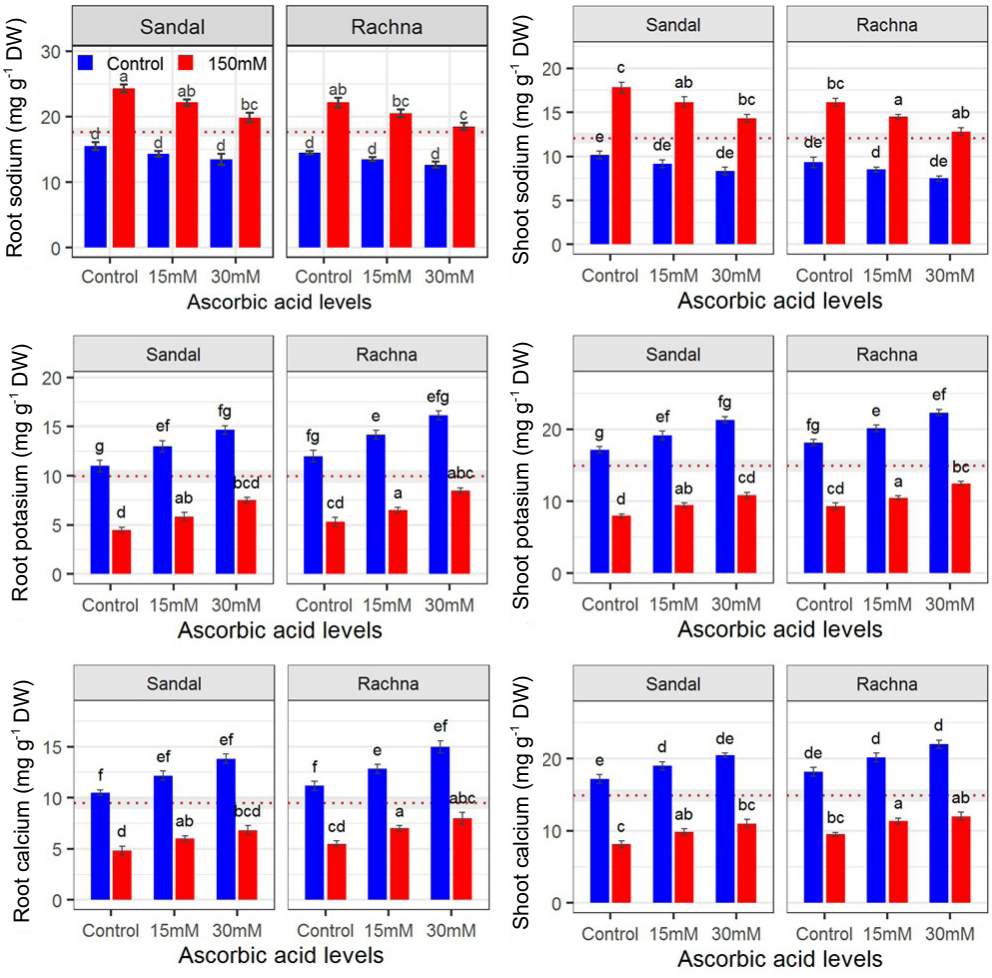

Fig. 5. Impact of exogenously applied ascorbic acid (AsA) on root and shoot ions of canola under saline conditions. Error bars represent the standard error (SE) across three replicates. For each parameter, means labeled with identical letters are not significantly different ($p < 0.05$). Blue bar – control (0 mM NaCl), red bar – salinity (150 mM NaCl). Other explanations as in Fig. 1.

further illustrated how treatments (combinations of varieties and AsA levels under salinity stress) are spatially separated along PC2, indicating that PCA effectively distinguished between stress responses. This separation aligns with observed physiological trends, such as increased AsA-mediated tolerance in certain treatments.

By integrating PCA findings with the physiological narrative, we demonstrate that PCA not only confirms the clustering of related traits but also supports the interpretation of treatment effects, particularly in distinguishing stress-responsive versus growth-promoting parameters.

4. DISCUSSION

Salinity has a negative impact on plant growth and development due to a variety of factors, including reduced osmotic potential in the soil solution, nutritional imbalance and most importantly, ionic impacts (Akhter *et al.,* 2022; Chaudhary *et al.,* 2024). Salt stress drastically decreased the morphological attributes such as the length of root and shoot, dry weight of root and shoot, fresh weight of root and shoot in both canola varieties. Growth inhibition under saline environments is driven by a combination of osmotic, nutritional, and structural disturbances. Osmotic stress leads to a reduction in cellular contents, thereby restricting water uptake and metabolic efficiency (Shahzad *et al*., 2019). This limitation further constrains tissue development and differentiation, as metabolic energy is diverted towards ionic regulation rather than morphogenesis (Maqsood *et al*., 2023). Salinity also induces nutritional imbalance by disrupting the uptake and allocation of essential ions, creating physiological deficiencies (Rasheed *et al*., 2024). In parallel, excessive ionic stress compromises membrane stability through lipid peroxidation and leakage, impairing compartmentalization and signaling processes (Alinia *et al*., 2022). Foliar spray of ascorbic acid (AsA) reduced the adverse effects of salinity and enhanced the morphological attributes in both varieties under both saline and non-saline conditions. Similar outcomes were also observed in pepper

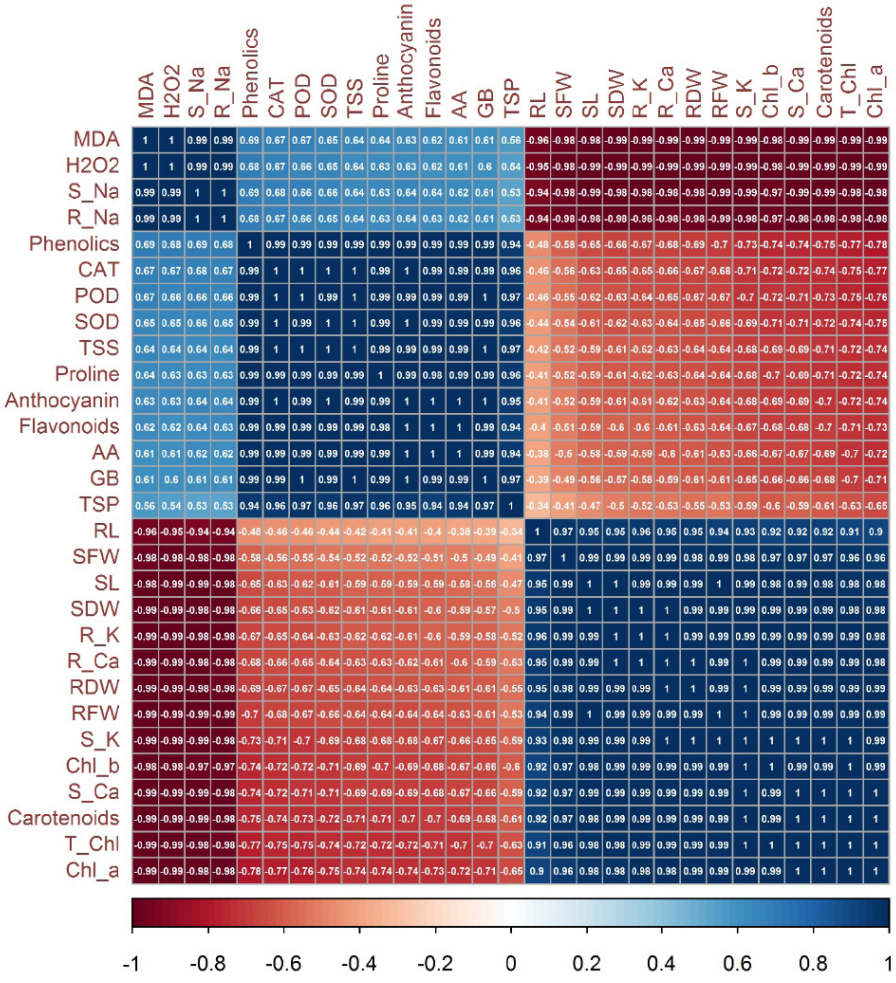

Fig. 6. Pearson's correlation analysis of 29 studied parameters showing r-values; absolute value of ±0.823 found significant at $p<0.001$ for 10 degree of freedom. So, any correlation in the correlogram below -0.823 and above 0.823 is declared as significant ($p<0.001$). Parameters have been ordered according to the first principal component (FPC).

(Khazaei *et al.,* 2020). Ascorbic acid (AsA) serves as a central component of the plant antioxidant defense system, particularly under salt-induced oxidative stress. It enhances stress tolerance by sustaining the activity and synthesis of antioxidant enzymes, thereby reducing reactive oxygen species (ROS) accumulation and associated cellular damage (Chen *et al*., 2021). In conjunction with other antioxidants, AsA preserves physiological and signaling pathways, maintaining redox balance critical for growth and development (Ishaq *et al*., 2021). Moreover, it influences a wide range of enzymatic functions by acting as both a cofactor and regulator, directly participating in redox reactions that mitigate oxidative stress (Azeem *et al*., 2023).

The photosynthetic pigments *i.e*. total chlorophyll, chlorophyll *a* and *b*, and carotenoids content drastically reduced under salt stress. Salt stress impairs photosynthesis by inactivating enzymes of both light and dark reactions, thereby restricting energy assimilation (Zhang *et al*., 2014). It further suppresses chlorophyll biosynthetic enzymes, limiting pigment accumulation essential for light harvesting (Attia *et al*., 2021; Iftikhar *et al*., 2024). Additionally, stomatal closure, thylakoid membrane damage, and reduced CO_2 uptake collectively disrupt electron transport and carbon fixation, leading to diminished photosynthetic efficiency (Sardar *et al*., 2023). The photosynthetic pigments improved in both varieties of canola by the foliar application of ascorbic acid (AsA) under both control and NaCl stress. Ascorbic acid protects photosynthetic machinery under salt stress by scavenging reactive oxygen species that otherwise inactivate photosynthetic enzymes (Borisova *et al*., 2012). Through this protective role, it preserves enzymatic integrity and chloroplast structure, thereby sustaining light-driven reactions. Consequently, AsA enhances photosynthetic efficiency and maintains relative chlorophyll content, supporting plant productivity under salinity (Celi *et al*., 2023).

Salt stress raised the levels of reactive oxygen species (ROS), including MDA and H_2O_2. Reactive oxygen species (ROS) function as dual agents, acting in signaling pathways

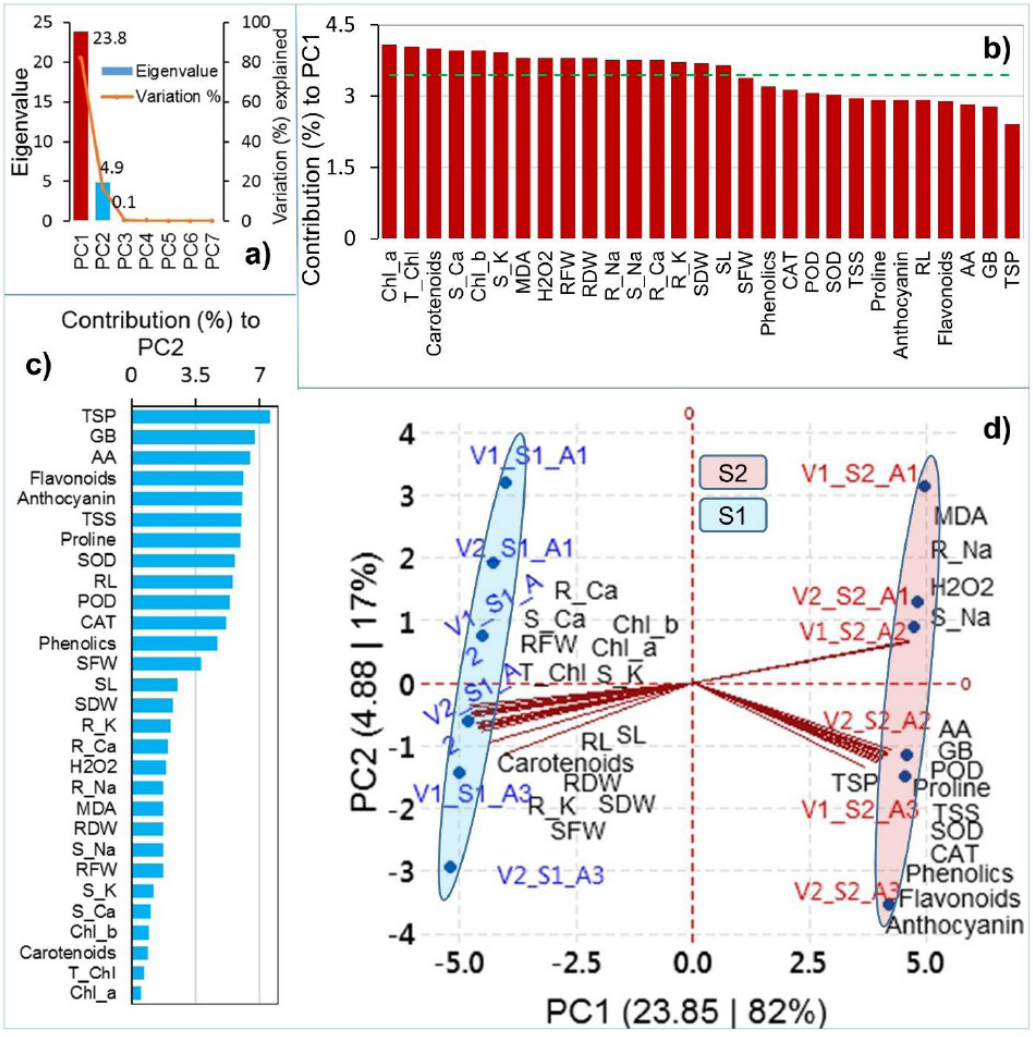

Fig. 7. Principal component analysis (PCA) of 29 variables across 12 observations. Eigenvalue analysis (a) revealed two components, PC1 and PC2 with eigenvalue of 23.8 and 4.9 respectively. Percentage of contribution of each parameter ordered at PC1 (b) and PC2 (c). Biplot of treatment in rows as 12 points and parameters in columns as 29 vectors (d) for two varieties (V1=Sandal, V2 = Rachna) at two levels of salinity (S1= 0 mM NaCl, S2= 150 mM NaCl) by the application of three AsA levels (A1= 0 M AsA, A2 = 15 mM AsA, A3= 30 mM AsA) shows their two dimensional spread along PC1 (eigenvalue 23.85 | variation explained 82%) and PC2 (eigenvalue 4.88 | variation explained 17%).

while simultaneously inducing oxidative stress that compromises cellular structures (Farooq *et al*., 2019; Naheed *et al*., 2022). Excessive ROS accumulation alters cell viability through membrane damage and metabolic disruption, impairing growth and survival. Moreover, they exert profound effects on the photosynthetic apparatus, disturbing electron transport and reducing efficiency (Xie *et al*., 2019). Additionally, ROS act as secondary messengers in response to stress and signaling pathways (Bano *et al.,* 2021). Under control and saline conditions, exogenous application of ascorbic acid significantly reduced the ROS produced in response to salt stress. Similar findings were observed in chickpea (El-Beltagi *et al.,* 2022). Ascorbic acid (AsA) serves as a vital antioxidant, directly scavenging reactive oxygen species to mitigate oxidative stress and protect cellular integrity (Dolatabadian and Sanavy, 2008). Beyond its direct role, AsA initiates antioxidant defense by modulating both enzymatic and non-enzymatic pathways, thereby sustaining redox homeostasis. This multifaceted function is particularly critical under salt stress, where oxidative pressure is elevated (Aydoğan *et al*., 2023).

It was observed that enzymatic antioxidants *i.e.* superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) considerably increased under salinity. By neutralizing excessive reactive oxygen species, antioxidant enzymes preserve membrane integrity and prevent lipid peroxidation, a key outcome of oxidative stress (Sardar *et al*., 2023). This protective mechanism not only stabilizes cellular structures but also maintains ion balance and metabolic activity. As a result, plants exhibit improved physiological

resilience and enhanced salt tolerance under stress conditions. Application of exogenous ascorbic acid increases the contents of SOD, POD and CAT under control and saline environment. Increased activity of superoxide dismutase, peroxidase, and catalase forms a coordinated defense system that detoxifies reactive oxygen species, thereby reducing oxidative pressure on cellular components (Hasanuzzaman *et al*., 2023). This enzymatic protection safeguards nucleic acids, proteins, pigments, and membranes from degradation, ensuring functional stability. Such regulation is vital for sustaining metabolic processes and structural integrity under salt stress.

Under saline conditions, both canola varieties showed an increase in total soluble sugar (TSS), total soluble proteins (TSP), and endogenous ascorbic acid (AsA). Same results were also found in saffron (Hamidian *et al.,* 2023). Accumulation of soluble sugars, proteins, and ascorbic acid contributes to osmotic adjustment, enabling cells to maintain turgor and water balance under salt stress (Perveen and Hussain, 2021). Beyond osmoprotection, these metabolites actively support metabolic pathways such as photosynthesis, ensuring sustained energy production (Rady *et al*., 2018). They also serve roles in storage and defense, strengthening plant resilience against salinity-induced damage (Jalili *et al*., 2023). Foliar application of ascorbic acid increased the TSS, TSP and endogenous AsA under both control and salt stress (Alves *et al.,* 2021). Ascorbic acid shields cellular organelles by scavenging excess reactive oxygen species, thus preventing oxidative damage to membranes and metabolic machinery (Barzegar *et al*., 2018). This protective role preserves essential physiological functions, allowing plants to maintain growth under adverse conditions. Consequently, AsA enhances stress endurance by reinforcing redox stability and cellular resilience (Noreen *et al*., 2021).

It was observed that the levels of flavonoids, anthocyanin and total phenolics were significantly increased under salt stress. Similar findings were also observed in lettuce (Zhang *et al.,* 2021). These essential secondary metabolites in plants protect against abiotic stresses by decreasing the production of reactive oxygen species (Moradbeygi *et al.,* 2020) and potentially preventing lipid peroxidation in stressed plants, hence promoting plant growth (Alizadeh *et al.,* 2021). Foliar spray of ascorbic acid enhanced the levels of flavonoids, anthocyanin and phenolics under both control and salt stress. Same results were also noticed in rice (Álvarez-Robles *et al.,* 2021). Exogenous ascorbic acid modulates cellular redox homeostasis, creating favorable conditions for the biosynthesis of flavonoids and phenolic compounds that strengthen antioxidant defenses (Wang *et al*., 2019). By reducing oxidative stress, AsA also stimulates anthocyanin accumulation, which functions as a protective pigment against salinity-induced damage (El-Hawary *et al*., 2023). This dual role highlights its capacity to regulate both metabolic pathways and stress-responsive mechanisms.

AsA modulates gene expression related to anthocyanin biosynthesis and redox regulation in various plants, including strawberry (Zhang *et al*., 2022), citrus (Liu *et al*., 2015), and tomato (Wang *et al*., 2020). These findings suggest that AsA plays a broader physiological role in enhancing plant metabolic homeostasis and general resilience beyond stress mitigation (Arabia *et al*., 2024). Moreover, AsA acts as a key redox regulator and cofactor for several enzymes involved in photosynthesis, hormone metabolism, and antioxidant regeneration (Raees *et al*., 2023). By maintaining cellular redox homeostasis, AsA ensures proper metabolic functioning and protects plant cells from oxidative damage under normal conditions, thereby supporting overall growth and resilience (El-Hawary *et al*., 2023; Parveen *et al*., 2024).

Under salt stress the concentrations of proline and glycine betaine significantly increased in both varieties. Proline stands out as a crucial osmotic protector, playing an essential role in enhancing resistance to salt challenges (Ahmad *et al.,* 2016). Its function involves regulating osmosis and preserving cell membranes (Dawood *et al.,* 2021). Proline is vital for preserving membrane structure by binding to phospholipids, altering the hydrated layer around biological macromolecules (Chourasia *et al.,* 2021). Glycine betaine levels rise with increased salt stress, enhancing plant stress tolerance by curbing lipid breakdown and maintaining osmotic balance (Khalid *et al.,* 2022). Under both control and salt stress, the foliar application of AsA increased the concentrations of proline and glycine betaine. Similar outcomes were also noticed in rapeseed (Hasanuzzaman *et al.,* 2023). The accumulation of proline and glycine betaine contributes significantly to salinity tolerance by enabling osmotic adjustment and protecting cells from dehydration stress (Yaqoob *et al*., 2019). These osmolytes also scavenge reactive oxygen species, preserve membrane stability, and support nutrient balance essential for metabolic functions. Furthermore, they facilitate ion compartmentalization while stabilizing proteins and enzymes, ensuring sustained physiological activity under saline conditions (Jalili *et al*., 2023).

Under NaCl stress the concentration of root and shoot Na^+ was considerably increased while the concentration of essential root and shoot K^+ and Ca^{2+} were drastically reduced. These results were also described in grape (Amorim *et al.,* 2023). Salt stress imposes dual challenges of osmotic imbalance and ionic toxicity, with the severity of ionic stress varying across tissues (Zeeshan *et al*., 2020). Excessive sodium accumulation in roots disrupts membrane permeability, leading to ion leakage and nutrient imbalance. This Na^+ toxicity further inhibits cell elongation and division, ultimately restricting plant growth and development (Noor *et al*., 2022). Calcium (Ca^{2+}) and potassium (K^+) are present in both the roots and shoots of plants, indicating their essential roles in supporting healthy cell metabolism and overall metabolic activity (Rasel *et al.,* 2021). The reduced absorption of vital nutrients, including

potassium and calcium ions, may be attributed to the unfavorable impacts of salt stress (Sadia *et al.,* 2023). The foliar application of AsA led to a significant reduction in the concentrations of root and shoot sodium ions (Na^+), while causing a remarkable increase in the levels of both shoot and root potassium ions (K^+) and calcium ions (Ca^{2+}). Similar findings were also observed in cabbage (da Silva *et al.,* 2021). Enhanced plant development under stress conditions is often attributed to improved nutrient uptake and strengthened antioxidant defense, which collectively reduce oxidative damage (Shabbir *et al*., 2022). In addition, better gas exchange, higher photosynthetic pigment levels, and regulated stomatal behavior optimize carbon assimilation and water use efficiency. These physiological adjustments work synergistically to sustain growth and productivity under adverse environments (Saheed and Qader, 2020; Hassan *et al*., 2021).

5. CONCLUSIONS

This study demonstrates that foliar application of ascorbic acid (AsA) significantly enhances the salt tolerance of canola by simultaneously improving physiological, biochemical, and structural defense mechanisms. The observed increase in photosynthetic pigments and the accumulation of osmolytes such as proline and glycine betaine indicate that AsA plays a critical role in sustaining cellular osmotic adjustment under NaCl stress. Moreover, the marked elevation of antioxidant enzyme activities (SOD, POD, CAT), coupled with reduced MDA and H_2O_2 levels, highlights the role of AsA in mitigating oxidative damage, preserving membrane integrity, and minimizing lipid peroxidation. The reduction in chlorophyll degradation further confirms its role in maintaining photosynthetic efficiency, thereby supporting plant growth and productivity under saline conditions. Taken together, these findings not only establish the efficacy of foliar AsA application as a practical strategy for improving canola performance in saline environments but also provide novel evidence linking osmolyte accumulation with antioxidant defense in mediating stress resilience. While our findings clearly demonstrate the physiological benefits of foliar AsA application in mitigating salt stress, the economic feasibility of large-scale application in field-grown canola remains an important consideration. Future studies should therefore incorporate agronomic and cost-benefit assessments to evaluate its practicality for sustainable crop production.

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