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INFLUENCE OF EXOGENOUS APPLICATION OF L-PROLINE ON GROWTH OF BARLEY (*HORDEUM VULGARE* L.) UNDER CADMIUM STRESS

MOAZMA BATOOL[1], QUM QUM NOSHAD[2], MUHAMMAD AJAIB[2], MUHAMMAD FAHEEM SIDDIQUI[3], HALEEMA SADIA[1] AND AYSHA KIRAN[1*]

[1]Department of Botany, University of Agriculture, Faisalabad-38040, Pakistan
[2]Department of Botany, Mirpur University of Science & Technology (MUST), Mirpur-10250 (AJK), Pakistan
[3]Department of Botany, University of Karachi, Karachi 75270, Pakistan
**Corresponding author's: aysha.kiran@uaf.edu.pk; mfsiddiqui@uok.edu.pk*

Abstract

Cadmium contaminated soil poses serious threat to human health and agriculture. Moreover, Cd toxicity poses significant challenges to the development of sustainable food production, particularly in crops like barley. Barley, due to its intrinsic ability to flourish in a variety of environmental stresses, grown on both small and large-scale farms. A pot experiment having completely randomized design with three replications was carried out to study the impact of foliar application of L-Proline (1mM) in reducing the toxic effects of cadmium (120µM) in two barley varieties, Jou-17 and Sultan-17. Cadmium stress results in remarkable reduction in plant biomass, leaf chlorophyll contents, carotenoids while improved the oxidative stress indicators like catalase, peroxidase and superoxide dismutase activity and tissue malondialdehyde and hydrogen peroxide contents. On the other hand, foliarly applied proline was effective in reducing toxicity of cadmium and also showed partial reversal of Cd stress by improving the growth attributes, Chlorophyll content, anthocyanins and carotenoids in barley. Of the two barley varieties, Jou-17 showed better performance under Cd stress in terms of enhanced leaf chlorophyll contents and less accumulation of H_2O_2 and MDA contents. Proline being an osmoprotectant infuses abiotic stress tolerance in barley plants under cadmium stress by reducing production of reactive oxygen species and enhanced accumulation of protein content.

Key words: Heavy metal stress, Cadmium, Antioxidant activity, Proline.

Introduction

After wheat, rice, and maize, barley (*Hordeum vulgare* L.) is the fourth most significant cereal crop in terms of economic importance. Out of the total worldwide barley production, three-quarter is used as an animal feed (Haas *et al*., 2019). In Pakistan, on area of 55 thousand hectares, 55 thousand tons of barley is produced (Anon., 2019). Barley contains all the essential nutrients in high quantity. Barley's increased dietary fiber and tocopherol contents are primarily responsible for its positive health effects. Barley is distinguished by having an average grain β-glucan concentration that is about ten times higher than that of wheat (Geng *et al*., 2021). Plant taxonomy named one of the variations of Graminae family as highland barley (HB). The nutritional functional components β-glucan, polyphenol, and arabinoxylon (Simic *et al*., 2019) which are principally responsible for HB's antibacterial, anticancer, and antioxidant effects, are exceptionally abundant in HB (Song *et al*., 2020).

Toxic heavy metal pollution in the environment, such as that caused by cadmium (Cd), arsenic (As), and chromium (Cr), has become a risk to human and agricultural sustainability (Luo *et al*., 2022; Nazir *et al*., 2011). These metals, which were present in agricultural soils, had a detrimental effect on crop growth and yields (Zheng *et al*., 2020). Among all heavy metals, cadmium is highly non-biodegrable, mobile and persistent element found in agricultural soils (Herliana *et al*., 2018; Moradi & Karimi, 2020; Nazir *et al*., 2013). Cd causes rapid alterations in barley's morphology. Alongwith these morphogenic changes Cd stress evokes several defence responses. Some of these reactions like an increase in antioxidant defense are common to all stressors, whereas others like the accumulation of phytochelatins (PCs) in the presence of toxic metals in soil are specific. PCs preserve cellular integrity and are crucial for heavy metal elimination. It is implicated in both metal sequestration and metal chelation in vacuoles and the cytosol, respectively (Faizan *et al*., 2024). Additionally, oxidative stress brought on by an excess of reactive oxygen species (ROS; $O^{2\bullet}$, H_2O_2, and OH) leads to unfavourable alteration in organelle structures. These ROS significantly disrupt all physio-biochemical processes connected to the functioning of plant cells, causing proteins oxidation, DNA damage, lipids peroxidation, and photosynthesis disruption (Verma *et al*., 2024).

Plants encountered destructive threats when exposed to heavy metals like cadmium. Heavy metals if uptake by plants cause severe damage to physiological and structural functions of plants (Kiran *et al*., 2024). Supplementing plants with osmoprotectants may be a workable method to increase their resistance to stress. By scavenging reactive oxygen species (ROS), preserving enzyme function, and stabilizing cellular structures, osmoprotectants also referred to as compatible solutes-assist plants in fending off oxidative and osmotic stress (Rasheed *et al*., 2024). Osmoprotectants have received a lot of attention because of their great effectiveness, simplicity of usage, low cost, and lack of requirement for specialized equipment. Osmoprotectants are tiny, highly soluble organic compounds having a neutral charge and minimal toxicity at physiological pH. They help plants to maintain cellular homeostasis under abiotic stress conditions (Sharma *et al*., 2024). Proline (Pro) is one of them that may be utilized to lessen the negative effects of oxidative stress on plants (Khalid *et al*., 2022) (Fig. 1). It act as a quencher of reactive oxygen species (ROS). When administered at the right concentrations, proline has been demonstrated to improve stress tolerance in a variety of food crops, such as cucumber, pea, rice, and sunflower (Shahid *et al*., 2022). Applying proline to stressed plants causes structural

alterations that increase the surface area of the roots to compensate for nutrition and water deficits. Additionally, it promotes root development and causes structural alterations in stressed plants' leaves and stems (Abdelaal *et al*., 2020). Osmoprotectants, especially proline, could be sprayed on leaves to mitigate the negative effects of copper stress. More proline was accumulated in the plant system, which improved the photosynthetic system and soluble protein (Noreen *et al*., 2018).

It alleviates heavy metal stress by acting as heavy metal chelator. Heavy metals like Cd become chelated with phytochelatins when proline is present, reducing their toxicity. Binding of proline to hydrogen bonds increased its ability to protect membrane's stability and also enhance stability of protein. Proline interacts with phospholipid head groups in cell membranes, reinforcing hydrogen bonding and maintaining membrane integrity (Umumararunga *et al*., 2024). Proline is an osmolyte but is also regarded as an antioxidant. During abiotic stress, external application of proline results in enhanced growth, endogenous proline content, antioxidant activities and photosynthetic activity which provide more protection to scavenged ROS, plasmalemma and stabilized proteins (Hosseinifard *et al*., 2022).

The objectives of present study were, first, to determine the effect of cadmium application on morphological, biochemical and physiological attributes of two barley varieties, second, to evaluate how proline ameliorate toxic effects of cadmium application on both varieties, finally, to determine which barley variety performed better under cadmium stress with proline application.

Material and Methods

Experimental setup: In order to investigate, effect of foliar application of proline on two barley varieties under cadmium stress, a pot experiment was carried out in Old Botanical Garden, University of Agriculture, Faisalabad under natural climatic conditions (Fig. 2) during the last week of November 2021. Barley varieties, namely Jou-17 and Sultan-17 were employed in current experiment. Seeds of both barley varieties were supplied by Ayub Agricultural Research Institute (AARI), Faisalabad, Punjab, Pakistan. Plastic pots having dimensions i.e., 25 cm, 22.5 cm, 20 cm were filled with thoroughly river washed (5 kg per pot) sand and 10 seeds of each variety were sown in them. After germination, thinning was carried out and seven seedlings per pot were maintained. To fulfill nutritional requirements of plants, full-strength Hoagland's nutrient solution having pH 5.5-6.5 was applied until the application of cadmium stress. Each pot received a 500 ml initial application of Hoagland's nutritional solution every two weeks, with successive applications using the same volume for the remaining intervals. After four weeks of germination, Cd stress (0 and 120 µM) application was done on barley plants along with Hoagland's nutrient solution. Next day of stress application, proline (Mol. Wt. 115.11; Sigma Aldrich, Waltham, MA, USA), was sprayed as foliar spray at the rate of 0 and 1 mM concentration. Cd stress was applied three times each after one week while foliar application was done only once. Harvesting of barley plants was carried out and plants were stored at -20 ˚C to record data for different morphological, biochemical and physiological attributes.

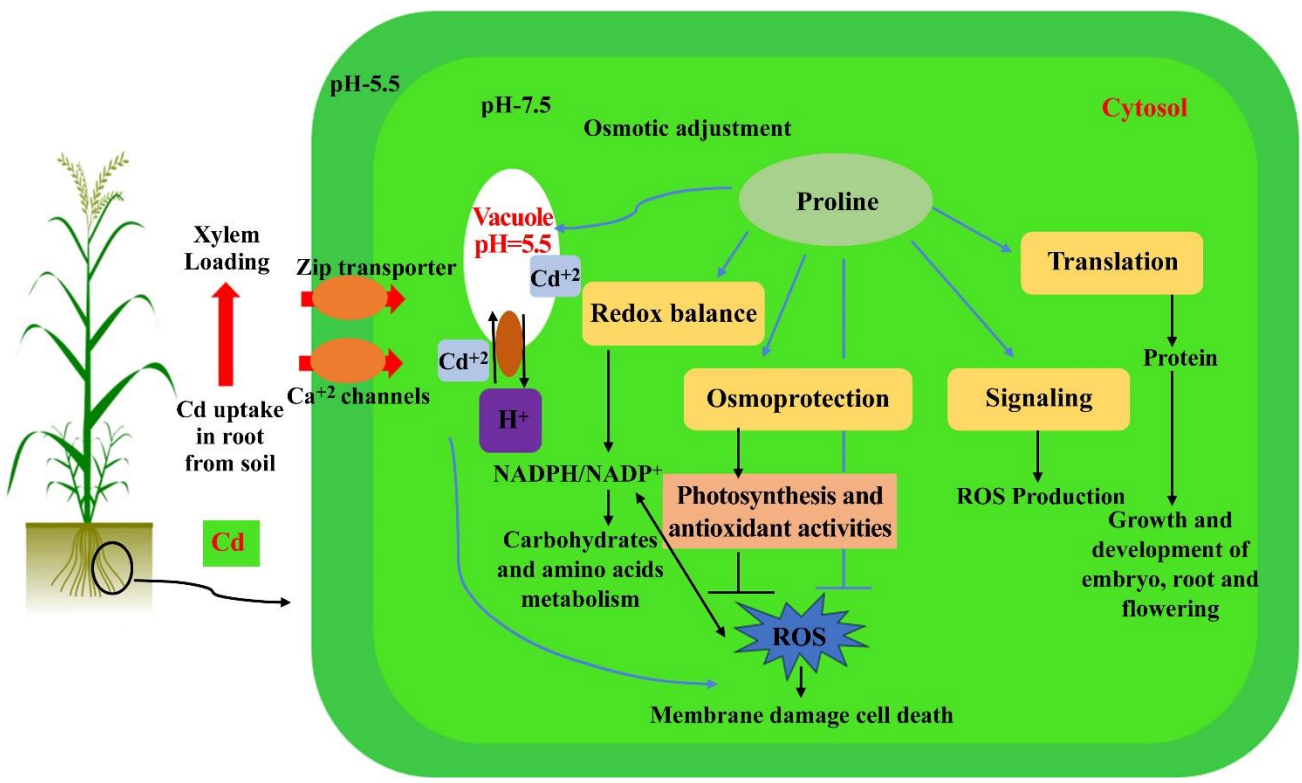

Fig. 1. Schematic diagram (reconstructed) showing Cd^{2+} uptake via Fe^{2+}/Zn^{2+} (ZIP family) and Ca^{2+} transporters, with vacuolar sequestration via Cd^{2+}/H^{+} antiport and proline-mediated detoxification and stress response (about proline adapted from Szabados & Savour, 2010).

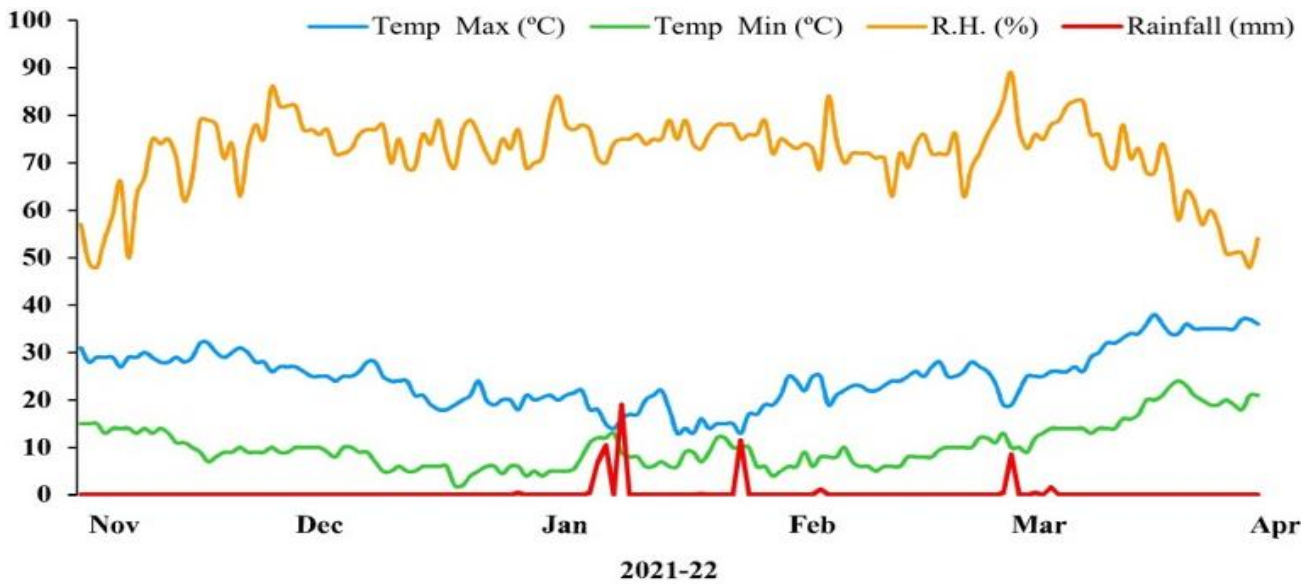

Fig. 2. Weather conditions during growing season 2021-2022.

Morphological attributes: In the midweek of February 2022, two plants from each replicate were harvested and immediately after harvesting a top-loading weighing balance was used to record the fresh weight of the plants. The lengths of the roots and shoots were measured in centimeters with the help of measuring tape. Then, these fresh plants were subsequently heated to 65°C in oven for five days, during which time dry masses were measured with the help of balance.

Determination of chlorophyll and carotenoids: For chlorophyll content and carotenoids measurement method of Arnon (1949) was followed. Using a pestle and mortar, fully grown fresh leaf (0.5 g) was ground in 5 mL of (80%) acetone, then left overnight for 10-12 h at room temperature. Afterwards, using a spectrophotometer (IRMECO U2020, IRMECO Gmbh, Schwarzenbeck, Germany), the extract from the following day was utilized to record absorbance at 480, 645, and 663 nm. Carotenoids and chlorophyll contents were measured in mgg^{-1} of fresh weight.

Determination of anthocyanin contents: With the help of the Stark & Wray (1989) method, anthocyanins were measured. 5 mL of acidified methanol was utilized to crush 0.2 g of fresh plant material. After that, the homogenate was heated in a water bath for one hour at 50°C. Absorbance was recorded at 535 nm using spectrophotometer (UV-Visible IRMECO U2020). The amount of anthocyanins was given as mgg^{-1} of fresh leaves.

Determination of enzymatic antioxidants activities in plant leaves: For determination of antioxidant activities, Phosphate buffer (5 mL) was used to grind fresh leaf (0.5 g) in chilled pestle mortar, which was then centrifuged at 12000 rpm for 12 minutes. After centrifugation, antioxidant enzymes were measured from supernatant using spectrophotometer (UV-Visible IRMECO U2020). Protocol of Chance & Maehly (1955) was followed to determine the activity of catalase. Reaction mixture (1.5 mL) containing phosphate buffer (950 µL), enzyme extract (50 µL) and hydrogen peroxide solution (500 µL) was added in cuvette to check absorbance (240 nm) from 0 to 90 seconds after every 30 seconds using spectrophotometer (UV-Visible IRMECO U2020). According to Giannopolitis & Ries (1977) activity of superoxide dismutase was assayed. In quartz cuvette, the reaction mixture contained distilled water (150 µL), nitrobluetetrazolium (50 µL), phosphate buffer (250 µL), methionine (100 µL), triton X (100 µL), enzyme extract (50 µL) and riboflavin (50 µL). The prepared mixture was then placed under fluorescent lights for 15 minutes to start the reaction. Absorbance at 560 nm was measured using spectrophotometer (UV-Visible IRMECO U2020). For POD assay, protocol of Chance & Maehly (1955) was applied. In cuvette, reaction mixture (1mL) contains enzyme extract (50 µL), phosphate buffer (750 µL), guaiacol (100 µL) and hydrogen peroxide solution (100 µL). Absorbance was recorded at 470 nm for 0s, 30s, 60s and 90s.

Determination of non-enzymatic antioxidants activities in barley plants: Method given by Bradford (1976) was applied to quantify total soluble proteins. For this, 0.25 g of fresh leaf sample was grinded in phosphate buffer (5ml) using chilled pestle and mortar, then grinded sample was centrifuged at 12,000 rpm for 10 minutes. Supernatant (5ml) and Bradford reagent (5ml) was taken in test tubes. Vortex samples and by using a spectrophotometer absorbance were checked at 595 nm. Protocol proposed by Hamilton & Van-Slyke (1943) with little modification was used to determine total free amino acids. Add 0.5 mL of 10% pyridine, 0.5 mL of 2% ninhydrin and 0.5 mL of already prepared enzyme extract in test tubes. Place test tubes rack in water bath for 30 min at 50°C. Then remove the test tubes from water bath and make the volume of solution upto 25 mL by adding distilled water. For anthocyanin extraction, procedure was performed

according to Stark & Wray (1989). Crushed 0.2 g of leaf sample was added in test tubes along with 2 mL acidified methanol. These test tubes were then heated in water bath at 90°C for 60 min. After removing from water bath, these samples were used for recording absorbance at 535 nm using a spectrophotometer.

Method of Kim *et al*., (1999) was used for extraction of flavonoids concentration from Barley leaves. To extract the plant material, 0.1 g of fresh leaf sample was grinded in 2 mL of 80% acetone. 0.5 ml plant extract and 2ml of distilled water was added in test tubes. Then after 5 minutes 0.6 mL of 5% sodium nitrate, 0.5 mL of 10% Aluminium chloride and after 1 min add 2 ml of NaOH. And after wait of 1 min add 2.4 ml of distilled water. Then readings were recorded at 510 nm with the help of spectrophotometer. Method proposed by Mukherjee & Choudhuri (1983) was applied for estimation of Ascorbic acid. 0.1 g of fresh leaf sample was grinded in 5 mL of 6% TCA solution. Reaction mixture was centrifuged for about 5 minutes and supernatant was separated. In test tubes, grinded extract (1mL), 2% DNPH (2ml) and 1 drop of thiourea was added. Water bath the solution containing test tubes for 20 minutes. Allow them to cool after removing from water bath. At the end, add 2.5 mL of 80% sulphuric acid. By using spectrophotometer, optical density was determined at 530 nm.

Determination of reactive oxygen species (MDA and H_2O_2): Health & Packer's (1968) thiobarbituric test was used to detect MDA levels. Fresh fully grown leaf was ground (0.25 g) in a cooled mortar using 2 mL of 0.1% TCA. For 12 minutes, the homogenate was centrifuged at 12000 rpm. The 1 mL of supernatant and 1 mL of TCA, which contained 0.5 g of thiobarbituric acid, were combined. In a water bath reaction mixture is heated for 1 hour at 75°C. Absorbance was recorded at 532 nm and 600 nm using spectrophotometer (UV-Visible IRMECO U2020). 5% TCA was used as a blank. Velikova *et al*., method (2000) was applied to measure the levels of H_2O_2. Fresh leaf was ground (0.25 g) in a cooled mortar with 2 mL of 0.1 percent tricarboxylic acid (TCA). Centrifuging the homogenate at a speed of 12000 rpm for 12 minutes. The supernatant (0.5 mL) and potassium phosphate buffer (0.5 mL) were combined with potassium iodide (1 mL) to react. The absorbance at 390 nm was measured using a spectrophotometer (UV-Visible IRMECO U2020). TCA (0.1 %) at was used as a blank.

Mineral Ion analysis (Na^+, Ca^{+2}, K^+): Allen, Gimshaw & Rowland (1996) was employed to check ions uptake by barley plants. 0.1 g of oven dried samples of both roots and shoots were crushed and added in digestion flask containing 5 mL of H_2SO_4. Place these flasks overnight at dark place. After this, flasks were heated on hot plate at 150°C followed by addition of H_2O_2 until solution becomes transparent. Samples were filtered and then diluted. The concentration of different ions (Na^+, K^+, Ca^{+2}) was estimated by using Flame Photometer (Sherwood flame Photometer-410, Sherwood Scientific Ltd. Cambridge, UK).

Statistical analysis

Three replications of each treatment were used in the entirely randomized design of the experiment. Analysis of variance was used to identify differences between various factors that were significant at p=0.05 (ANOVA). The statistical analysis was done using "Statistix 8.1" software. The Pearson correlation coefficients between biochemical parameters and ionic contents of barley were also calculated using RStudio software.

Results

Effect of cadmium and proline on morphological and physiological attributes: Cadmium application results in marked decrease in plant biomass (Table 1) compared to control. More obvious decrease 6.03% was observed in Sultan-17 under stress conditions indicating that Jou-17 was more tolerant to stress than Sultan-17. However exogenous proline application significantly overcome the toxic effects of cadmium on plant morphology. Proline as a foliar spray results in greater plant fresh and dry weight and shoot and root lengths by 31.63%, 25.64%, 4.9%, 6.6% respectively except for Sultan-17 fresh and dry weight in which weight decreases by 36.5% and 27.4% respectively when both proline and Cd was applied. Cd application cause remarkable degradation of chlorophyll (*a* and *b*) contents in both barley varieties (Table 1). Higher chlorophyll *a* was observed in Jou-17 than that of Sultan-17 under control conditions. Massive decrease of Chlorophyll *a* and *b* contents were in Jou-17 by 23.6% and 33.1% respectively under stress conditions when compared with reference plants. Foliar application of proline increased Chlorophyll *a* contents in Sultan-17 by 34.04% and Chlorophyll *b* contents in Jou-17 by 29.02% (Table 1). Carotenoids reduced under Cd stress application by 11.2 % in Sultan-17 while Jou-17 showed less reduction (6.98%) when compared with control. Foliarly applied proline alleviated the decrease of carotenoids in both varieties. This effect was more remarkable in Jou-17 (14.28%) than in Sultan-17 (6.25%).

Effect of Cd and Proline on Enzymatic antioxidants and Reactive oxygen species: As compared to control, antioxidant activities (SOD, Catalase, POD) increased in both barley varieties by 52.8%, 35.68% and 31.9% respectively under cadmium application (Fig. 3A, B, C). But this increase was more obvious in Sultan-17 than Jou-17 under stress conditions in case of SOD. Activities of catalase, SOD and POD increased when foliar spray of proline was applied in Sultan-17 while Jou-17 did not show any significant change in these antioxidant enzyme activities except in CAT activity. Higher amounts of MDA were present in Jou-17 (38.27%) and while in Sultan-17 H_2O_2 is present in large quantity (8.75%) when treated with cadmium (Fig. 3D, E) Both varieties showed varied response with respect to these variables. For instance, higher endogenous levels of MDA and H_2O_2 were observed in a Jou-17 in stress conditions as compared with control. Exogenous application of proline results in marked reduction in reactive oxygen species levels (MDA and H_2O_2) by 3% and 3.63% in both varieties.

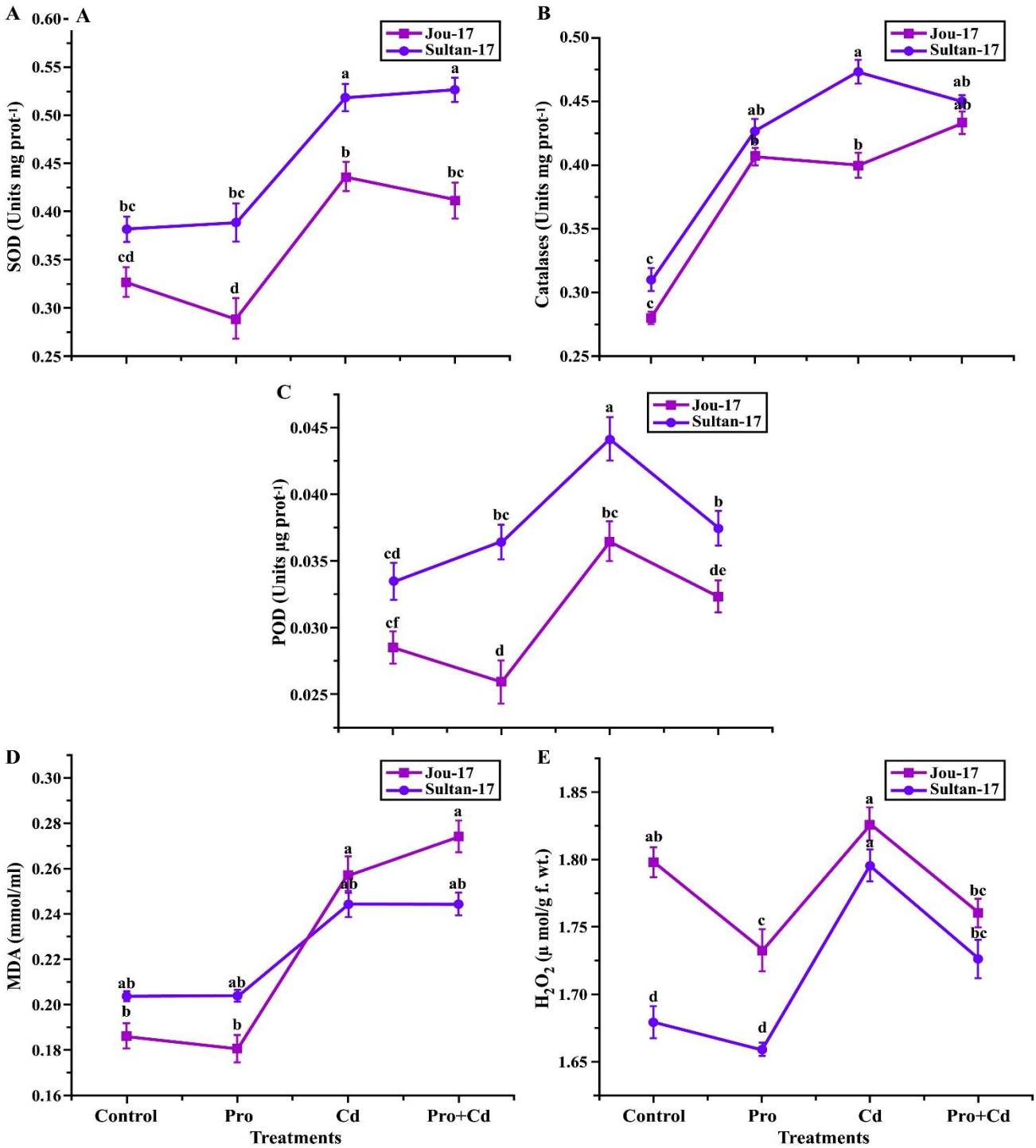

Fig. 3. Superoxide dismutase (SOD) activity (Units mg prot^{-1}), Catalase activity (Units mg prot^{-1}), peroxidase (POD) activity (Units µg prot^{-1}), MDA (mmol/ml) and H_2O_2 (µmol/ g f. wt.) in leaves (A, B, C, D, E) influenced by Cd stress and foliar application of proline in two barley varieties. ±SE shown as error bars. Significant differences between different treatments are shown by different letters at $p \leq 0.05$.

Effect of Cd and proline on non-enzymatic antioxidants: Non-enzymatic antioxidants of both varieties, Jou-17 and Sultan-17 showed greater significant behavior under cadmium stress application (Fig. 4). Cd treatment decreased the total soluble sugar contents (21.07%) in Jou-17 and (17.37%) in Sultan-17 compared to control plants (Fig. 4A). When compared to control, there was an increase in the contents of free amino acids (7.97% and 6.64%), flavonoids (20.41% and 6.52%) ascorbic acid (6.81% and 7.94%) and anthocyanins (9.17% and 12.17%) under cadmium exposure (Fig. 4B, C, D, F). Total soluble proteins decreased (5.68%) in Jou-17 while in Sultan-17 their contents enhanced (5.58%) under Cd treatment (Fig. 4E). On the other hand, foliar application of proline mitigates the cadmium stress by enhancing total soluble proteins (12.08%) in Jou-17 while in Sultan-17 foliar application did not show significant increment in total soluble protein contents. Application of proline substantially increased ascorbic acid contents (25%, 20%), flavonoids (24.69%, 7.27%) and anthocyanins (9.21%, 12.47%) respectively in Jou-17 and Sultan-17 as compared to control plants.

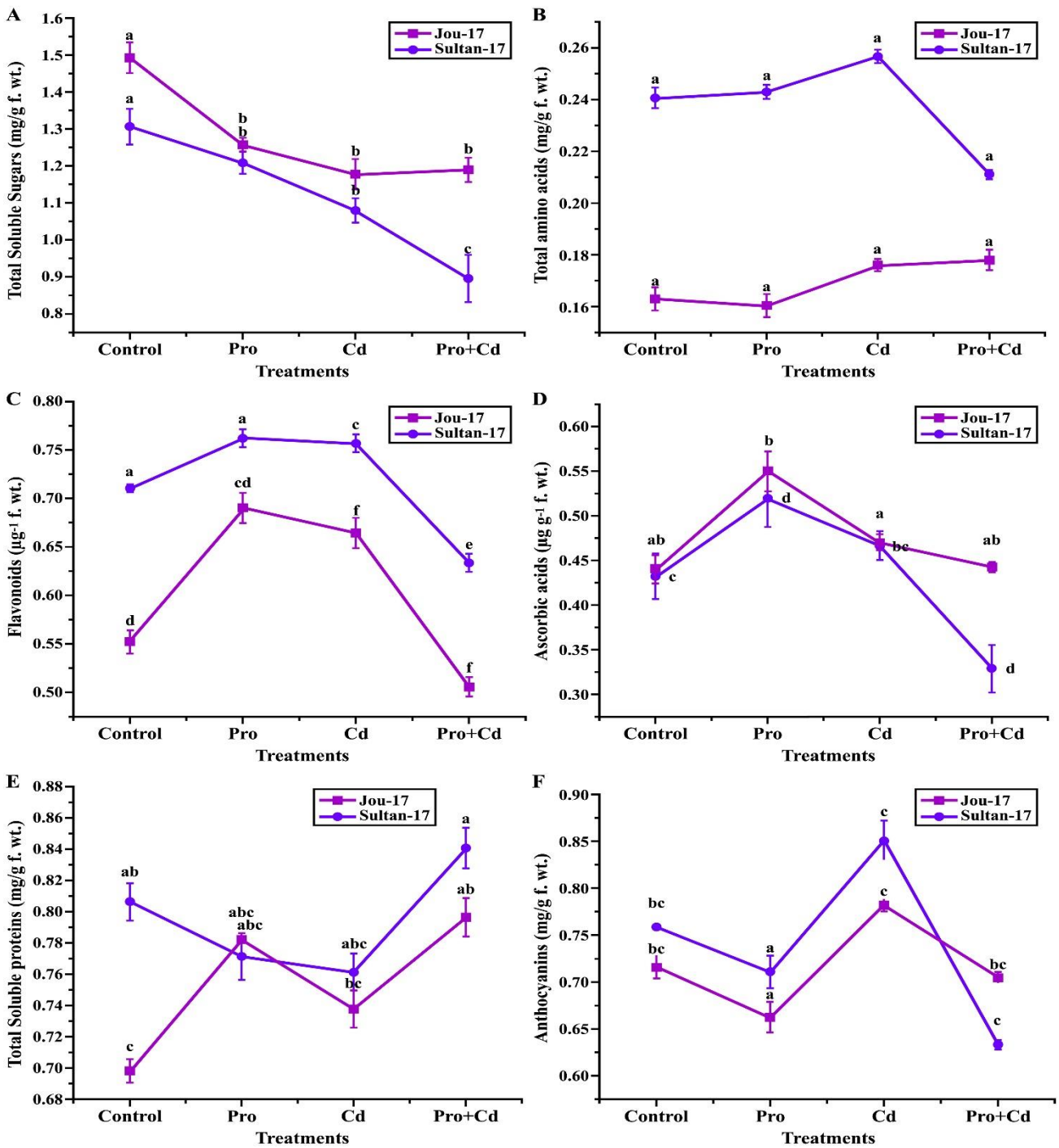

Fig. 4. The effect of Cadmium and proline on two barley varieties on Total soluble sugars (A), Total amino acids (B), Flavonoids (C), Ascorbic acid (D) and Total soluble proteins (E) and Anthocyanins (F). Error bars on points show standard error between mean of three treatments. Similar letters on points indicates that there is no significant difference (p<0.05) among means of three treatments while different letters indicates significant difference (p<0.05) according to LSD.

Effect of Cd and proline on mineral ion content (K^+, Ca^{+2}, Na^+): Mineral Nutrients including potassium and calcium were significantly reduced under cadmium stress. Ca^{+2} concentration decreased in shoots (16.66%, 24.28%) and roots (21.42%, 5.88 %) of Jou-17 and Sultan-17 when exposed to cadmium. Similarly, shoots (5.12%, 8.93%) and roots (48%, 5.55%) of both varieties showed significant reduction in K^+ contents under stress conditions. (Fig. 5A, B, C, D) Ca^{+2} increased in shoots (21.87%, 4.65%) and roots (16.66%, 17.64%) of both Jou-17 and Sultan-17 as compared to non-stressed plants when proline was foliarly applied. Likewise, K^+ contents increased in both varieties in shoots (2.5%, 18.6%) and also in roots (21.87%, 4.65%) respectively under proline application. Imposition of Cadmium stress significantly increased shoot (13.25%, 17.33%) and root (18.75%, 16.27%) Na^+ contents of two barley varieties, Jou-17 and Sultan-17 in comparison with control plants. However proline application tended to reduce root and shoot Na^+ contents in Jou-17 (21.87%, 2.40%) and also root and shoot Na^+ contents in Sultan-17 (4.65%, 8%) as compared to control plants (Fig. 5E, F).

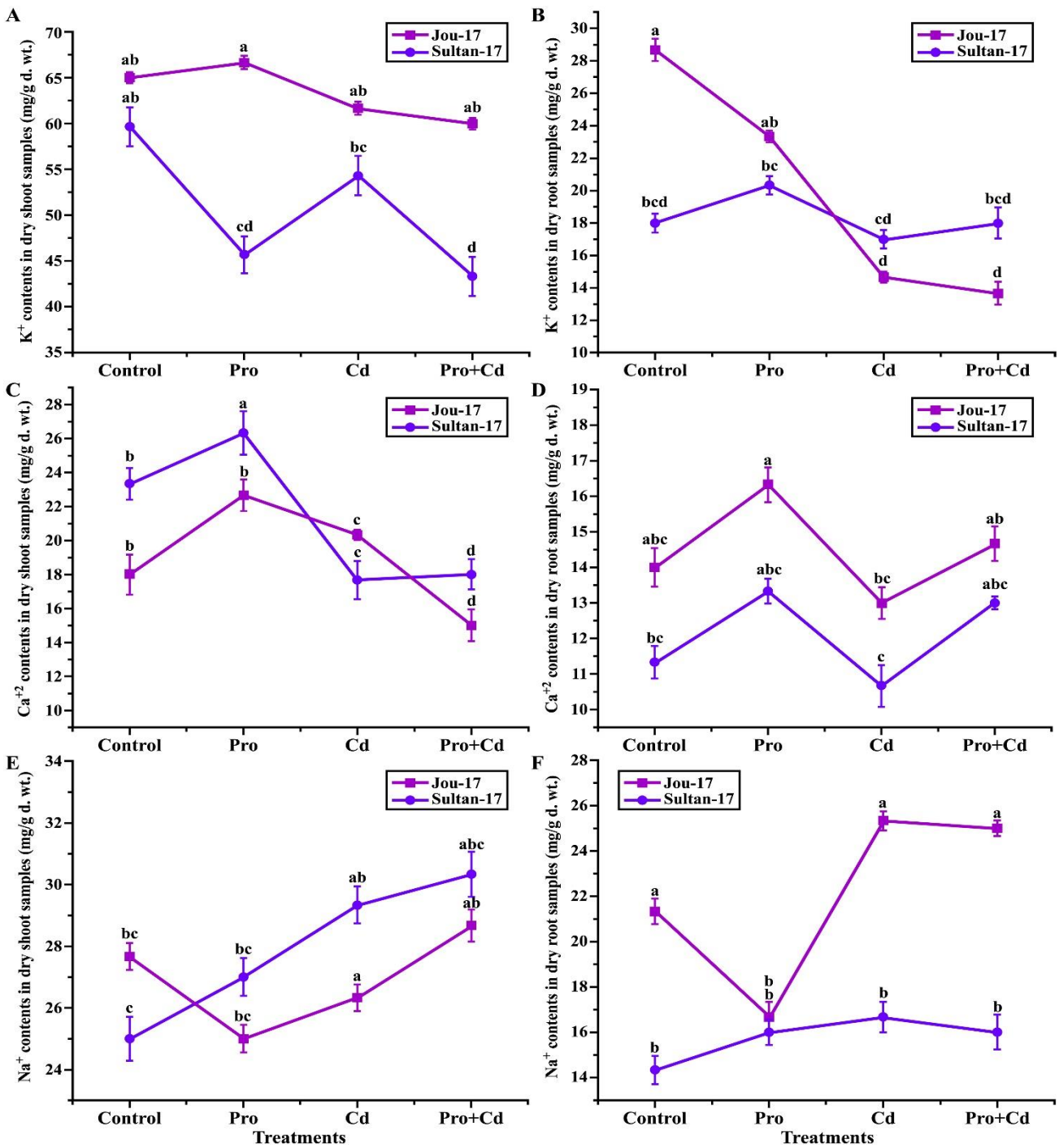

Fig. 5. Changes of the dry shoot K^+ ions (A), dry shoot Ca^{+2} ions (C), dry shoot Na^+ ions (E), dry root K^+ ions (B), dry root Ca^{+2} ions (D), dry root Na^+ ions (F). Error bars on points show standard error between mean of three treatments. Similar letters on points indicates that there is no significant difference (p<0.05) among means of three treatments while different letters indicates significant difference (p<0.05) according to LSD.

Table 1. Shoot fresh weight, root fresh weight, root dry weight, shoot dry weight, shoot length, root length, Chl. *a* and Chl. *b* values of two barley varieties when exposed to cadmium stress and foliar application of proline.

Varieties	Treatments	Shoot fresh weight (g)	Root fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)	Shoot length (cm)	Root length (cm)	Chl. a (mg g^{-1}f.wt)	Chl. b (mg g^{-1}f.wt)
Jou-17	Control	13.4±0.6^{bc}	0.66±0.00^{a}	0.93±0.03^{bc}	0.13±0.00^{a}	55±1.90^{a}	13.5±0.64^{a}	0.76±0.01^{ab}	0.55±0.01^{c}
	Cadmium (Cd)	12.3±0.7^{bc}	0.63±0.04^{bc}	0.94±0.05^{bc}	0.11±0.1^{a}	53.1±1.64^{bc}	12.3±0.68^{a}	0.58±0.1^{c}	0.37±0.01^{d}
	Proline	15.6±0.61^{ab}	0.67±0.02^{a}	1.2±0.08^{a}	0.16±0.00^{a}	58.5±1.11^{a}	14.3±0.68^{a}	0.81±0.02^{a}	0.71±0.03^{a}
	Cd + Proline	11.92±0.34^{c}	0.627±0.04^{a}	0.8±0.04^{cd}	0.13±0.00^{a}	53.2±1.24^{bc}	13.3±0.68^{ab}	0.65±0.0^{c}	0.44±0.00^{d}
Sultan-17	Control	16.9±0.8^{a}	0.68±0.02^{bc}	1.14±0.06^{ab}	0.15±0.00^{a}	53.8±0.56^{ab}	15±0.68^{ab}	0.61±0.01^{c}	0.63±0.03^{b}
	Cadmium (Cd)	11.2±1.1^{cd}	0.64±0.02^{bc}	0.71±0.03^{cd}	0.13±0.01^{a}	51±0.44^{b}	12.3±0.46^{a}	0.61±0.00^{c}	0.40±0.00^{d}
	Proline	14.1±0.98^{abc}	0.90±0.02^{a}	0.87±0.02^{cd}	0.17±0.00^{a}	55.1±0.67^{bc}	16±0.44^{a}	0.82±0.01^{a}	0.70±0.04^{b}
	Cd + Proline	8.12±0.09^{d}	0.57±0.00^{bc}	0.65±0.08^{d}	0.12±0.01^{a}	46.03±1.47^{d}	15±0.67^{ab}	0.76±0.00^{ab}	0.59±0.02^{ab}

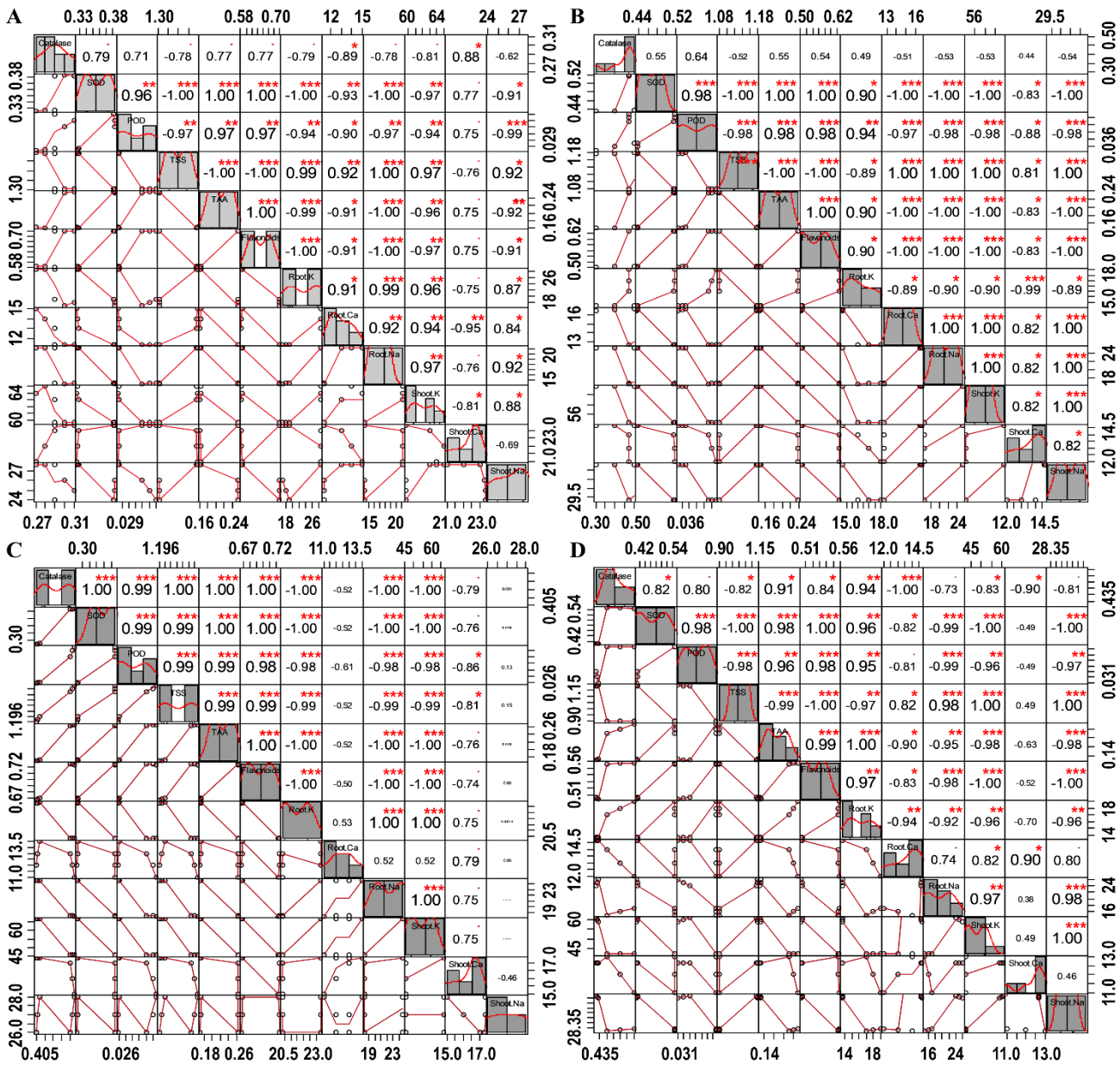

Fig. 6. Pearson correlation matrix for the relationship of biochemical attributes with ionic contents of both varieties of barley (a) Biochemical attributes and ionic contents under control conditions (b) Biochemical attributes and ionic contents under stress conditions (cadmium) (c) Biochemical attributes and ionic contents under foliar application (Proline) and (d) Biochemical attributes and ionic contents under stress conditions when proline was applied.

Correlation of biochemical attributes and ionic contents of both barley varieties: Comparison of biochemical attributes with ionic contents of both varieties of barley under control conditions showed positive correlation of SOD with total amino acids (TAA) (R^2=1) and flavonoids (R^2=1). Total soluble sugars (TSS) strongly correlate with root K (R^2=0.99) and root Na (R^2=1). Total amino acids showed positive correlation with flavonoids (R^2=1). Root K showed positive correlation with root Na (R^2=0.96) (Fig. 6a).

Comparison of biochemical attributes with ionic contents of both varieties of barley under stress conditions showed positive correlation of SOD with POD (R^2=0.98), total amino acids (R^2=1), flavonoids (R^2=0.98). POD strongly correlates with total amino acids (R^2=0.98), flavonoids (R^2=0.98). Total soluble sugars showed positive correlation with root Ca (R^2=1), root Na (R^2=1) and shoot K (R^2=1). Total amino acids positively correlate with flavonoids (R^2=1). Root Ca showed positive correlation with root Na (R^2=1), shoot K (R^2=1) and shoot Na (R^2=1) (Fig. 6b).

Comparison of biochemical attributes with ionic contents of both varieties of barley under foliar application of proline showed positive correlation of catalase with SOD (R^2=1), POD (R^2=0.99), TSS (R^2=1), TAA (R^2=1) and flavonoids (R^2=1). SOD showed positive correlation with POD (R^2=0.99), TSS (R^2=0.99), TAA (R^2=0.99) and flavonoids (R^2=1). POD showed positive correlation with TSS (R^2=0.99), TAA (R^2=0.99) and flavonoids (R^2=0.98). TSS showed positive correlation with TAA (R^2=0.99) and flavonoids (R^2=0.99). Root K showed positive relationship with root Na (R^2=1) and shoot K (R^2=1). Root Na positively correlates with shoot K (R^2=1) (Fig. 6c).

Comparison of biochemical attributes with ionic contents of both varieties of barley under stress conditions with foliar application of proline showed positive correlation of SOD with POD (R^2=0.98), TAA (R^2=0.98) and flavonoids (R^2=1). POD showed positive correlation with flavonoids (R^2=0.98). TSS showed positive correlation with root Na (R^2=0.98), shoot K (R^2=1) and shoot Na (R^2=1). TAA showed positive correlation with flavonoids (R^2=0.99) and Root K (R^2=1). Root Na showed positive correlation with shoot Na (R^2=0.98). Shoot K with Shoot Na (R^2=1) (Fig. 6d).

Discussion

Plants develop oxidative stress under Cd toxicity as indicated by many reports (Rizwan *et al*., 2016; Zouari, 2016). Under heavy metal stress, proline accumulates. Cd induced growth inhibition is mitigated by proline (Meena *et al*., 2019). In current experiment, cadmium application significantly reduced growth attributes (Plant fresh and dry weight, shoot and root length). As reported earlier by Man *et al*., (2019) that Cd caused toxic symptoms like necrosis, stunted growth and chlorosis in barley tissues. Massive decrease in plant biomass and length was also noticed. Rady *et al*., (2019) also noticed decrease in plant *Phaselous vulgaris* growth attributes like plant fresh and dry weight, shoot and root length when exposed to different Cd concentrations. Cadmium decreased the elongation rate of cell thus interferes with plant growth and development. Cd disrupts cell wall structure and loosening**,** which is essential for elongation (Deng *et al*., 2024). Foliar application of proline ameliorates the toxic impact of cadmium by improving growth attributes. Better growth of both barley varieties under foliar spray of proline could be linked with its role to act as a subcellular compartment stabilizer. It maintains cell turgor and redox potential under Cadmium stress (Meena *et al*., 2019).

L-proline mitigates cadmium (Cd) stress in plants through multiple interconnected biochemical mechanisms, primarily involving osmoprotection, metal chelation, antioxidant defense, and redox homeostasis. As an osmoprotectant, proline accumulates in response to Cd stress, stabilizing proteins, membranes, and cellular structures while maintaining water potential and ion homeostasis (Zulfiqar & Ashraf, 2023). Its ability to chelate Cd ions reduces their free cytoplasmic concentration, facilitating sequestration into vacuoles and minimizing Cd interference with essential cellular functions. Additionally, proline directly scavenges reactive oxygen species (ROS) generated by Cd-induced oxidative stress and enhances the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), thereby preventing lipid peroxidation and cellular damage (Wang *et al*., 2022).

In current study, chlorophyll *a*, *b* contents, anthocyanins and carotenoids of both barley varieties were negatively affected by Cd application. Massive decrease in photosynthetic pigments like chlorophyll, anthocyanins and carotenoids was observed in *Groenlandia densa* under Cd application (Yilmaz & Parlak, 2011). Amari *et al*., (2017) elaborated according to their findings that Cd stress results in growth retardation and depletion of chlorophyll contents among barley cultivars. Rucinska *et al*., (2016) stated that cadmium stress inhibits the synthesis of chlorophyll. This was more prominent on surface of leaves rather than mesophyll cells. Chlorophyll is produced when protochlorophyllide and light interact. Cadmium (Cd) toxicity interferes with the de novo synthesis of important metabolic precursors**,** including levulinic acid**,** which is involved in chlorophyll and heme biosynthesis. Cd-induced disruption of these pathways leads to impaired growth, photosynthesis, and overall plant metabolism (Vila-Santa *et al*., 2021). Cadmium prevents the conversion of protochlorophyllide to chlorophyll in the presence of light by hindering the activity of protochlorophyllide reductase (Bhardwaj *et al*., 2024). In contrast higher chlorophyll under foliar spray of proline might be due to the reason that chlorophyll gain its structure and start performing photosynthesis (Hayat *et al*., 2021).

A dramatic increase in antioxidant enzymes (CAT, POD, SOD) was noted in both barley varieties under cadmium stress which is in agreement with previous research (Man *et al*., 2019). Although in Sultan-17 more accumulation of catalase, SOD, POD indicating that it has more potential to cope with Cd stress by developing antioxidant defense mechanism. Only Cd application cause high levels of antioxidant enzymes. However when proline is applied along with cadmium it suppressed antioxidant enzymes concentrations. Furthermore there is a noticeable difference among two barley cultivars with respect to these parameters. Plants develop defense mechanism when heavy metal stress was applied. Current experiment indicates that cadmium application effects are more pronounced in terms of increased endogenous levels of MDA and H_2O_2 among both barley cultivars when compared with control. These results are in accordance with Tamas *et al*., (2008) who observed that barley root tips when exposed to Cd showed increased reactive oxygen species like MDA and H_2O_2. In response to stress, MDA levels are commonly measured as a marker of lipid peroxidation.

Soluble sugars play significant role in growth and development of plants. These are the products of photosynthesis and also play important role in whole process of photosynthesis. Soluble sugars maintain water potential of cell by reducing osmotic potential of cells thus maintaining normal metabolism of organisms (Abd Allah *et al*., 2017; Zhang *et al*., 2018). It was observed that in current experiment total soluble sugars contents decreased under cadmium stress in both varieties. This outcome might have been brought about by the destruction of chloroplasts and the accompanying reduction in photosynthesis when stress levels increased. To tolerate heavy metal (HM) stress**,** plants undergo significant metabolic reprogramming**,** utilizing soluble sugars as key resources to mitigate stress effects. These sugars play critical roles in osmotic adjustment, energy supply, ROS scavenging, and metabolic defense responses under heavy metal toxicity (Sharma *et al*., 2024). Many studies revealed that, Proline, when exogenously applied alleviate toxicity of heavy metals (Shahid *et al*., 2014). This can be explained in current investigation in which total soluble sugars increased in barley plants treated with proline. Because proline enhances tolerance against Cd stress by maintaining antioxidant defense system,

stabilizing cellular structures, and mitigating oxidative damage (Wang *et al*., 2022). Increase in soluble sugars contents when proline was exogenously applied was previously reported (Sattar *et al*., 2024).

The present study showed that Cd stress increased flavonoid contents in both varieties when compared with control. This is mainly due to reduced chlorophyll content that induced tolerance against heavy metal stress (Giannakoula *et al*., 2021). Flavonoids contain hydroxyl groups in their rings that can easily scavenge ROS produced after Cd exposure (Goncharuk & Zagoskina, 2016). Through ion chelation followed by vacuolar sequestration Flavonoids can detoxify metalloids (Ahammed & Yang, 2022). In present study, foliar spray of proline further increased flavonoids of both varieties under stress regimes. Flavonoids are the plants secondary metabolites, which act effectively, as scavengers of oxidizing molecules including singlet oxygen and free radicals (Ali *et al*., 2007; Ajaib *et al*., 2024).

In current investigation, Ascorbic acid contents increased in both barley varieties under Cd toxicity. As reported earlier, under Cd application concentration of ascorbic acid increased (Younis *et al*., 2015). Ascorbic acid being non-enzymatic antioxidant enzymes accumulate in leaves and act as a co-factor for many enzymes, supports ROS detoxification**,** and enhances stress tolerance mechanisms (Kumari *et al*., 2024). By acting as a reductant, ascorbic acid is essential in preventing oxidative damage to cellular metabolism (Alamri *et al*., 2018).

Results of present experiment showed that total soluble proteins (TSP) decreased in Jou-17 when treated with cadmium. Amri *et al*., (2016) also observed that when barley plants were treated with cadmium there is decrease in TSP. The inhibitory effects of Cd stress on TSP are well known (Younis *et al*., 2015). This is because protein synthesis is strongly damaged when cadmium is aggravated. In addition, under Cd stress, proteins which are involved in ATP activities declined, causing reduction in soluble proteins (Xu *et al*., 2016). While foliar application of proline showed increasing trend of TSP in both varieties in current investigation. Changes in protein contents as a result of exogenous proline correlated with increases in the internal content of proline suggesting that proline was taken up into the roots and transported to the shoots. This process highlights proline's dual function as an osmoprotectant and metabolic regulator under stress conditions like cadmium (Cd) toxicity, salt stress, and drought (Yan *et al*., 2024).

The present study demonstrated that Cd stress had negative effect on mineral nutrients (K^+ and Ca^{+2}) in both barley varieties while Na^+ contents enhanced under Cd application. These results are in agreement with Ullah *et al*., (2016). By maintaining physiological levels of mineral nutrients, plant development can be achieved. The observed variations in mineral nutrients clearly depict that exogenous application of proline improved K^+ and Ca^{+2} mineral contents in both barley varieties. Foliar proline was very effective in maximizing K^+ and minimizing Na^+ in wheat (Mahboob *et al*., 2016). Proline enhances ion uptake in roots for sustaining cell membrane H^+- ATPase activity.

Conclusion

According to our findings, it was concluded that heavy metal such as cadmium stress remarkably reduced the barley's growth while foliar application of osmoprotectant like proline was affective in improving plant's growth by overcoming cadmium stress adverse effects and by producing tolerance in plant.

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