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A COMPARISON OF EXOGENOUS PROLINE APPLICATION METHODS IN ENHANCING SALINITY TOLERANCE OF FLEX (*LINUM USITATISSIMUM* L.)

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Abstract

Proline (Pro) is one of the compatible solutes that play an important role against salinity through osmotic adjustment and protecting membranes in plants. The aim of the present study was to determine the alleviation of salinity stress by the application of proline in flax plant. Three flax genotypes Roshni, Chandni and Desi were tested against salinity, i.e. 0 mM NaCl (Control) and 180 mM NaCl. Two levels of proline 0mM and5 mM and were applied through three different mods, i.e. pre-sowing seed treatment, foliar spray and root growing medium. Salinity substantially decreased the growth and yield of flax plants by decreasing shoot fresh, photosynthetic pigments (Chlorophyll *a* and *b*) with a concurrent decrease in both shoot and root Ca^{2+} and K^{+}. A considerable increase in both shoot and root Na^{+}, increased level of antioxidants (catalase, peroxidase, superoxide dismutase) and total soluble proteins was also observed in flex plants exposed to salinity stress. Exogenous application of proline enhanced the growth and yield of flex plants. This was accompanied by increased shoot fresh weights and antioxidants with a parallel increase in Ca^{2+} and K^{+} contents of shoot and root tissues. At the same time, a substantial decrease in both shoot and root Na^{+} was noted under exogenously applied proline. Among all proline levels, 5mM concentration showed the most improvement in growth and yield of salinity stressed flax plants. As observed from PCA model, foliar application of proline was the most effective in alleviating the adverse effects of salinity stress compared with rooting or water sprayed flex plants.

Key words: *Linum usitatissimum*, Proline, Salinity, Biochemical analysis.

Abbreviations: RCa: Root calcium, **SNa:** Shoot sodium, **SCa:** Shoot calcium, **SOD:** Superoxide dismutase, **SK:** Shoot potassium, **TSP:** Total soluble proteins, **CAT:** Catalase, **RFW:** Root fresh weight, **SFW:** Shoot fresh weight, **SWP:** Seed weight per plant, **NPP:** Number of pods per plant, **RK:** Root potassium, **Chl a:** Chlorophyll a, **Chl b:** Chlorophyll b, **POD:** Peroxidase.

Introduction

Linum usitatissimum L. 2n = 30 (flax) it is the member of family Linaceae which consist of about 22 genera and 180 species widely distributed in six continents (Vromans, 2006; McDill *et al*., 2009). This is also known as flax seed or linseed. Since more than 5000 years, flaxseed was often used in food as well as textile fiber (Wakjira *et al*., 2004). Nowadays, the plantation area of flaxseed has expected to exceed 2.6 million hectares, and the significant flaxseed growing nations around the globe are China, Canada, Ethiopia, India, and the United States, respectively. Canada seems to be the world's largest producer, accounting for roughly 80% of global flaxseed production (Singh *et al.*, 2011).

With an amount of 55 percent in the oil, it is an excellent source of omega-3 fatty acid and linolenic acid (Oomah, 2001). Flaxseeds are edible and rich source of α-linolenic acid (ALA). In industrial uses, it is commonly known as the linen and is also used for the manufacturing of linoleum and printing inks, clothes, rag-based bags, table linen in Western countries. Linseed is an important potential source of minerals (2.4%), lipids (37.1%), carbohydrates (28.9%), dietary fiber (4.8%), protein (20.3%), phenolic compounds and vitamins (Singh *et al*., 2011). Flaxseed is very well known for the high proportion of α-linolenic acid (ALA), and it is thought to be the most advanced source of omega-3 fatty acids (PUFAs), after soybean, fish oil, maize, and marine algae (Wu *et al*., 2019). These complex series of poly-unsaturated fatty acids, including such docosapentaenoic acid eicosapentaenoic acid and docosahexaenoic acid, are thought to have a wide range of vital functional and physiological properties, including the development of cranial nerves, improve immunity, enhancing memory, and boosting the body's metabolism of essential fatty acids, as well as the prevention of myocardial injury and cerebral thrombosis (Burdge & Calder, 2005).

Flax is grown for dual purpose (stem for fiber and seeds for oil). Therefore, it is essential to increase flax seed production per unit area which could be attained by using stress tolerant and high yielding varieties (Ibrahim, 2009). The human population is expected to reach 8 billion by 2025. To avoid food shortages, saline soils must be renewed and managed in order to meet the food demands of a continuously increasing human population (Ladeiro, 2012). Crop production is also not growing rapidly in accordance with food demand. In most cases, lower crop production is caused by various abiotic stresses. Reducing crop losses due to various environmental stresses is a major concern in order to meet rising food demands (Shanker & Venkateswarlu, 2011). The various biotic and abiotic stresses, such as cold, salt stress, temperature, and dry spells, have a deleterious influence on the growth, yield, and biomass production of major food crops by up to 70% (Ahmad *et al*., 2012). Salt stress affects over 800 million hectare land world-wide (Munns, 2005). Salinity, in particular, reduces the quality and quantity of crop production (Saito *et al*., 2008).

Among different salts in soil causing soil salinity, sodium chloride (NaCl) is most effective and abundant. Due to its ability to fight essential nutrients it causes nutrient deficiency (Tester and Davenport, 2003; de Azevedo Neto *et al*., 2006). Plants exposed to highly saline conditions may also encounter ROSs (reactive oxygen species) such as hydroxyl radicals (OH^{-}), superoxide (O_2^{-}), and hydrogen peroxide (H_2O_2). These ROSs (reactive oxygen species) have higher ability to damage plant tissues due to highly

reactive properties (Implay, 2003). Osmotic potential (Ψ_π), turgor pressure (Ψ_p), and leaf water potential (Ψ_w) are all reduced by salinity. Soil salinity levels decrease soil water potential, resulting in water shortfall or osmotic pressure. Salt stress may cause ionic stress and reduces the vital role of K and Ca by accumulating toxic Cl^- and Na^+ (Munns & Tester, 2008).Salt content disrupts cellular homoeostasis and disrupts normal ion compartmentalization (Ashrafi & Nejad, 2017). Increased levels of ROSs in cells accelerate enzyme and protein deterioration, as well as lipid peroxidation (MDA) and then further degrades the ETC (electron transport system), PSII system, and membrane structure (Li *et al*., 2017). Subsequently, it raises the levels of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA). Plants are protected from the injurious effects of ROSs by producing different antioxidants i.e. enzymatic and non-enzymatic. The most important antioxidant enzymes include peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) (Rehman *et al*., 2019).

Osmotic adjustment is essential to maintaining turgor pressure, which really is necessary to maintain production efficiency, development, and yield. To regulate salinity effects in plants, numerous methods were used. Plants synthesize a diverse range of osmolytes, including proline (Pro), glycine betaine (GB), alanine, soluble sugars, glutamic acid, valine, serine, isoleucine, arginine, glycine, aspartic acid, and non-protein amino acids like pipecolic acid, ɣ-amino-butyric acid, citrulline, and ornithine. One of the well-known changes in plant metabolism in response to salt stress is proline synthesis. It has been reported that Pro is significantly promoted in response to saline environments, and proline accumulation thus is linked to be plant salt tolerance (Ahmad *et al*., 2016).Among different forms of L-proline accounts for less than 5% of total free amino acids in plants under normal conditions. The concentration of the amino acid increases up to 80% in various plants under various types of stress. Proline (Pro) is an abundant amino acid, osmoprotectant, and signaling molecule that assemble in the cell cytosol, stabilizing and protecting the plant cell wall, various protein enzymes, and proteins. The high quantities of proline scavenge ROS, restrict metabolic reactions during stress by up-regulating membrane protein, and keeps cell solute homeostatic control (Zhang & Dai, 2019).

Exogenous application of proline compound on crop production can improve salinity tolerance (Heuer, 2010). Considering the growth regulatory effect of proline, it was hypothesized that exogenously applied proline should have a positive effect on morpho-physiological and bio-chemical attributes in flax under saline condition. This study intended to draw the relationship between exogenously applied proline and morpho-physiological changes under non-saline and saline condition. Additionally, the determination of the effective mode of proline application among pre-sowing, foliar application and root growing medium was also in the preview of the present study.

Material and Methods

Experimental setup: The Flax seeds were acquired from AARI (Ayub Agriculture Research Institute Faisalabad). The experiments were carried out in sand culture as root media with 3 varieties of Flax (Roshini, Chandini and Desi) at 2 priming conditions (priming of seeds with water and 5 mM proline) under two salinity levels (0 mM and 180 mM). In experiments there were three modes of application of proline were used i.e. seed priming, foliar and rooting medium. The experiments were carried out in CRD (completely randomized design) with four replications.

Application of proline treatments: For seed priming treatment, the seeds of Flax genotypes were primed for 8 hours in proline solution (5mM) or in distilled water for hydro-primed at room temperature. The seeds of rooting medium or foliar spray mods were sown without any prior treatment. For foliar application, the proline was dissolved in distilled water with Tween twenty and sprayed to completely wet all leaves. For rooting mode proline solution was directly applied in growing medium mixed with Hoagland solution.

Plantation of flex seeds: Sand was washed with water before filling in plastic pots. Each pot was filled with 7kg pre-washed dry sand. Twenty (20) seeds (unprimed or primed as per plan)were sown in each pot and irrigated with Hoagland's nutrient solution which was replaced after every 8-10 days (Hoagland and Arnon, 1950). Ten plants per pot were left to grow after thinning. The required NaCl levels were applied by mixing in Hoagland's solution to create the desired salinity levels. The drained Hoagland solution was regularly monitored for maintenance of salinity levels during course of study.

Shoot and root fresh weight: Plants were uprooted after 50 days of treatment application and washed with tape water followed by running water. Roots and shoots were separated and used for morphological data recording. Fresh weight of shoots and roots was determined with the help of electrical balance immediately after harvest and was expressed in grams (g). For dry weights, plants were wrapped in paper bags, oven dried at 65ºC till constant dry weight before taking dry weight measurements.

Estimation of photosynthetic pigments: Important photosynthetic pigments chlorophyll *a*, *b* and carotenoids were estimated by the method reported by Arnon (1949) and Davis (1976), respectively. A 0.1g fresh leaf sample was taken and 80% acetone (5ml) was added for extraction through grinding. The optical densities of this extract were determined by using spectrophotometer (IRMECO-U2020) at wavelength of 663, 645 and 480 nm. These optical densities were then used in formula for the determination of chlorophyll *a*, *b* and carotenoids.

Extraction of antioxidative enzymes: For extraction of antioxidants, a 0.25 g of fresh leave was grounded in 5 ml of phosphate buffer (50 mM having pH 7.8) with the help of pestle and mortar under low temperature. The mixture was then centrifuge at 12000×*g* for 10 min. and supernatant was stored at -20°C for antioxidant assay.

Determination of superoxide dismutase (SOD): Superoxide dismutase (SOD) was measured by using the method of as purposed by Giannopolitis & Reis (1977).

Reaction mixture containing 50 µl Nitro blue tetrazolium (NBT), 50µl riboflavin, 100µl methionine, 250µl phosphate buffer (pH 7.8), 400µl water, 100 µl triton-x and 50µL enzyme extract in cuvette was kept for 20 min. under white florescent light. The absorbance was then recorded at 560 nm by using spectrophotometer (IRMECO-U2020). One unit of SOD activity was defined as the amount of enzyme that inhibited by 50% NBT photo reduction. SOD activity was recorded by using formulae:

$$\text{SOD activity (Unit mg prot.}^{-1}) = \{(A_{ck}-A_c) \times V\} + \{0.5 \times A_{ck} \times W \times V_t)\}$$

where A= OD at spectrometer, A_{ck} OD for control tube under light condition, V= Total volume of buffer solution used to extract they enzyme, W= Weight of sample, V_t = Amount of enzyme extract used in reaction solution to test SOD (Dixit *et al*., 2002).

Determination of peroxidase (POD): Peroxidase was evaluated by the method as devised by Chance & Maehly (1955). Reaction mixture was maintained by using the 750µl potassium phosphate buffer (pH 7.8), 100µl of 20mM guaiacol, 100µl of 40mM H_2O_2 and 50µl enzyme extract in a cuvette. The increase absorbance of the mixture was then recorded at 470nm by using Spectrophotometer (IRMECO-U2020) after interval of every 30 Sec. for 120 Sec. One unit of POD activity was defined as the amount of enzyme. One unit of enzymatic activity was defined as the amount of the enzyme that caused a change of 0.001 in absorbance per min. POD activity was recorded by using formulae:

$$\text{POD activity (Unit µg prot.}^{-1}) = (\text{Activity} \times A \times V \div A)/E \times W$$

Determination of catalase (CAT): CAT activity was estimated by the method used by the Chance & Maehly (1955). A 1.9 ml of 50 mM potassium phosphate buffer (pH 7.0), 1000 µl of 5.9 mM H_2O_2 and 100 µl of enzyme extract was added while maintaining the volume of the reaction mixture up to 3 ml. The decrease in absorbance was noted on spectrophotometer after every 20 Sec. for 120 Sec. at 240nm on spectrophotometer (IRMECO-U2020). The CAT activity was recorded by using formulae:

$$\text{CAT activity (Unit mg prot.}^{-1}) = (\text{Activity} \times A \times V \div A)/E \times W$$

Total soluble protein contents (TSP): For estimation of soluble protein, Bradford method (1976) was used in which 1.9 ml of G250 Coomassie Brilliant Blue dye (freshly prepared) was added to 100 µL of the extract. The absorbance of this mixture was then checked at 595nm. The final conc. of TSP was determined by a standard curve developed from Bovine Serum Albumin (BSA).

Nutrients analysis: The protocol of Wolf (1982) was followed to estimate mineral elements like Na, Ca and K by acid digestion. Dried material 0.1g of shoot and root was taken in digestion flask and concentrated $H_2SO_4$was added in it overnight at room temp. The very next day that digestion flask was placed at hot plate for heating at 350°C.After 30 min, 1ml of 35% H_2O_2 was added. Heating process continued till the mixture become colorless. Then it was filter and diluted with distilled water and make volume up to 50ml. The filter was used for determination of Na^+, Ca^{2+} and K^+ by using flame photometer (PFP 7, Jenway, Japan).

Yield parameters: The tagged plants were left to grow till maturity. After harvesting of matured and ripened flax plant, the number of pods per plant and seed weight per plant was recorded.

Statistical analysis: Data of all parameters was analyzed through MSTAT-C (CoHort software, Berkeley CA) following Steel *et al*., (1997). A 4 factor (variety, proline levels, salinity and mode of application), Analysis of Variance (ANOVA) was run. The LSD values were calculated for each mode separately by running a three ways ANOVA (Pro, Var, Sal) and used to place letters of significant among different modes. Heatmap clustering and Principal Component Analysis (PAC) was visualized in R Studios (R version 4.1.3.)

Results

Shoot fresh weight was significantly affected by 180 mM salinity level in all flax varieties. An increase in shoot fresh weight was noted under proline application. This effect was clear in Roshni as compared to Chandni and Desi under hydro-priming and saline condition. The maximum increase in shoot fresh weight was observed in Roshni variety when 180 mM salinity was applied under pre-sowing mode of application. The minimum reduction was noted when 5mM proline was applied as pre sowing treatment. Significant $p \leq 0.05$ genotypic differences were also observed for shoot fresh weight (Table 1). Overall Roshni performed better than the Chandni and Desi under 5mM proline application (Fig. 1A). Salinity stress had more adverse effect on the Desi variety than the Roshni and Chandni. Flex variety Roshni produce more fresh weight than the other varieties in all modes (Fig. 1B).

Photosynthetic parameters: The Chl *a* content decreased significantly in all varieties with the maximum in cv. Chandni grown under saline conditions. The concentration of Chl *a* pigment substantially increased when plants were applied with 5mM proline. Here, the maximum increase was exhibited by cv. Roshni. In foliar application, the maximum increase was observed in Chandni variety applied with proline (Fig. 2A). In rooting medium the maximum increase was measured in cv. Roshni. In rooting medium, cv. Chandni showed the highest Chl *a* contents when applied under saline condition (Table 1 and Fig. 2A).

Statistical effect of proline on chlorophyll b content of flax plants are shown in Table 1. Chlorophyll *b* content was significantly lowered with the increasing level of salinity stress in all varieties of flax (Roshni, Chandni and Desi). Application of proline in all modes (seed priming, foliar and rooting) significantly affected the chlorophyll b content. The minimum increase was observed in Desi variety in hydro-primed plants. The maximum increase in Chandni variety was observed as compared to the Roshni and Desiunder foliar application mode of proline application in 5 mM concentration (Fig. 2B).

Table 1. Mean squares for various growth and physiological attributes, and ionic contents of the three flex genotypes applied with different modes of proline under saline conditions.

SOV	df	SFW	RFW	Chl a	Chl b	SOD	POD	CAT	TSP
Varieties (V)	2	97.60***	66.17***	51.84***	62.05***	1.72 ns	10.52***	12.48***	3.20*
Salinity (S)	1	35.24***	49.80***	22.34***	0.06ns	14.86 ***	0.12ns	0.85ns	7.66**
V*S interaction	2	4.71*	2.77ns	3.17*	3.47*	6.65**	4.21*	17.16***	1.15ns
Modes (M)	2	22.66***	39.00***	64.98***	5.93**	5.06 **	47.33***	0.75ns	65.19***
V*M interaction	4	6.93***	1.74ns	9.42***	4.88**	1.30 ns	23.84***	8.21***	4.98**
S*M interaction	2	10.88***	6.19**	63.57***	17.74***	11.14 ***	31.87***	2.54ns	23.17***
V*S*M interaction	4	5.4***	4.33**	5.37***	5.53***	4.69 **	30.57***	15.40***	3.36*
Proline (P)	1	0.22ns	2.80ns	0.26ns	4.09*	5.86 *	89.03***	3.04ns	0.32ns
V*P interaction	2	5.22**	10.82***	0.91ns	1.80ns	1.04 ns	4.17*	8.66***	1.58ns
S*P interaction	1	0.39ns	4.25*	8.70**	8.002**	2.41 ns	4.51*	15.21***	0.0008ns
V*S*P interaction	2	2.23ns	3.80*	0.18ns	0.83ns	1.95 ns	15.19***	0.87ns	0.14ns
M*P interaction	2	8.43***	5.72**	1.20ns	2.62ns	2.4 ns	2.66ns	25.86***	0.51ns
V*M*P interaction	4	5.28***	3.22*	4.40**	2.05ns	1.56 ns	7.05***	7.29***	0.38ns
S*M*P interaction	2	1.69ns	3.15*	1.26ns	3.26*	0.60 ns	8.45***	24.63***	0.92ns
V*S*M*P interaction	4	0.90ns	1.21ns	3.21*	6.98***	1.42 ns	20.52***	49.68***	0.47ns
Error	108	173.52	1.14	0.002	0.0005	11.92	1.35	0.02	25.47
Total	143	843.22	5.12	0.009	0.002	25.19	9.72	0.12	81.24
	df	**SNA**	**RNa**	**SK**	**RK**	**SCa**	**RCa**	**NP**	**SWP**
Varieties (V)	2	23.79***	3.97*	65.64 ***	2.94ns	3.35*	3.42 *	29.89***	29.89***
Salinity (S)	1	589.09***	585.10***	331.42***	27.49 ***	1.59ns	7.56 **	701.98***	701.98***
V*S interaction	2	5.49**	0.29ns	17.65***	4.87 **	0.97ns	1.54ns	27.67***	27.67***
Modes (M)	2	12.83***	4.83**	11.70***	20.76 ***	1.20ns	7.51 ***	43.98***	43.98***
V*M interaction	4	4.77**	0.54ns	36.04***	4.39 **	4.10**	4.68 **	16.86***	16.86***
S*M interaction	2	13.20***	6.85**	343.87***	38.17 ***	3.17*	6.90 **	2.13ns	2.13ns
V*S*M interaction	4	3.37*	2.85*	76.98***	6.34 ***	3.61**	0.88ns	5.14***	5.14***
Proline (P)	1	6.17*	5.48*	2.44ns	0.37ns	0.02ns	2.60ns	148.70***	148.70***
V*P interaction	2	9.30***	4.24*	4.14*	4.94 **	2.50ns	0.57ns	1.03ns	1.03ns
S*P interaction	1	0.52ns	3.07ns	49.51***	0.44ns	4.86*	5.48 *	0.96ns	0.96ns
V*S*P interaction	2	13.16***	1.06ns	106.49***	1.20ns	1.53ns	0.82n	7.38***	7.38***
M*P interaction	2	2.35ns	0.09ns	33.40***	16.63 ***	1.53ns	0.78ns	13.09***	13.09***
V*M*P interaction	4	4.55**	0.98ns	23.83***	1.21ns	7.518***	0.63ns	7.17***	7.17***
S*M*P interaction	2	2.79ns	2.46ns	16.68***	2.017ns	0.23ns	2.52ns	3.81*	3.81*
V*S*M*P interaction	4	0.33ns	1.65ns	81.20***	6.03 ***	1.08ns	2.98 *	1.36ns	1.36ns
Error	108	5154.84	12154.68	933.36	17195.3	392.04	2091.06	94.67	0.59
Total	143	43992.3	87037.47	22147.86	62294.24	757.53	4036.55	1174.33	7.32

Abbreviations: SNA: Shoot Na^+; RNA: Root Na^+; SK: Shoot K^+; RK: Root K^+; SCa: Shoot Ca^{2+}; RCa: Root Ca^{2+}; NO: N of pods per plant; SWP: Seed weight per plant; *** = Significant at p<0.01, ** = Significant at p<0.01, * = Significant at p<0.05, ns = Not significant

Antioxidants: The statistical analysis of the data for anti-oxidants is presented in Table 1. Salinity stress significantly affected SOD contents in all flax varieties (Table 1; Fig. 3A). Proline application significantly ($p \leq 0.05$) enhanced SOD activity in all flex varieties. The maximum increasein SOD activity was seen in Roshni variety when proline was applied in rooting medium. The minimum SOD activity was seen in Chandni variety under pre-sowing proline mode. Overall, the most effective mode of proline application was through the pre-sowing and root growing medium (Fig. 3A). Proline application significantly enhanced POD activity. Notably, the Chandni variety exhibited maximum POD activity when subjected to 180mM salinity and through root medium application. Application of 5 mM proline through the rooting medium significantly increased POD activity ($p \leq 0.05$) under saline conditions. Overall, Chandni performed better than Roshni and Desi varieties in both controlled and salt-stressed conditions (Table 1; Fig. 3B).

Proline application significantly affected CAT activity, with all modes showing significant differences ($p \leq 0.05$). The highest CAT activity was observed in the Chandni variety when 5 mM proline was applied via foliar spraying. Moreover, foliar application of proline showed better results in Roshni and Desi varieties under both saline and non-saline conditions, indicating that foliar application was more effective than root treatment and seed priming (Table 1 and Fig. 4A). The imposition of 180 mM salinity significantly impacted the total soluble protein (TSP) content in all flax genotypes (Table 1, Fig. 4B). The protein content showed an increasing trend with salt application, particularly in the hydro-priming mode and with 5 mM proline application in all varieties. Notably, the Chandni variety exhibited the maximum increase in protein content when treated with 180 mM salinity under the pre-sowing mode of application. In contrast, the minimum value was observed in the same cultivar under control conditions in the rooting mode. The data suggests that proline application through the priming mode was more effective than the rooting and foliar application modes (Fig. 4B).

Shoot and root ionic contents: The sodium (Na^+) ion content in the shoots of flax plants significantly increased ($p \leq 0.05$) in all genotypes under salinity stress (Table 1; Fig. 5A). The highest Na^+ content was observed in the Desi variety when 180 mM NaCl was applied in the rooting

medium, compared to other modes (Fig. 5A). In contrast, the lowest sodium ion content was found in the Roshni variety when 5 mM proline was applied via foliar spraying. Additionally, 180 mM NaCl salinity significantly ($p \leq 0.05$) increased root Na^+ in all flax genotypes (Fig. 5B). The highest value was observed in the Desi variety under pre-sowing mode and control conditions in the root growing medium. Here, proline application significantly decreased root sodium in all cultivars, with the most significant reduction occurring in the root growing medium under both saline and non-saline conditions (Fig. 5B).

The potassium (K^+) ion content in shoots significantly increased when proline was applied to saline-stressed plants (Table 1, Fig. 6A), which was significantly decreased under stressed conditions. The highest shoot K^+ content was observed in the Roshni variety under control conditions in the root growing medium (Fig. 6A). Application of 5 mM proline enhanced K^+ ion content in all flax genotypes in the pre-sowing mode. Proline application also increased K^+ ions in roots, with pre-sowing and foliar modes being more significant than others. Seed priming mode was highly significant, enhancing K^+ ions in roots, with the maximum value observed in Chandni variety compared to Roshni and Desi when proline was applied at 5 mM concentration in seed priming mode (Table 1, Fig. 6B).

The imposition of 180 mM salinity had a significant impact on the calcium (Ca^{2+}) content in all flax varieties (Table 1, Fig. 7A). Proline application in the rooting medium and control conditions substantially increased Ca^{2+} ions in shoots. However, in foliar and priming modes, Ca^{2+} ions increased when salinity was imposed (Fig. 7A). The maximum increase was observed in the Desi variety when 5 mM proline was applied through priming mode. Salinity (180 mM) decreased Ca^{2+} ions in roots, except in a few cases (Table 1 and Fig. 7B). Proline application enhanced Ca^{2+} content in roots, except in Roshni and Chandni varieties in the root growing medium. The highest Ca^{2+} ion content was observed in roots when 5 mM proline was applied in seed priming mode. However, foliar and pre-sowing modes of proline application were more effective in alleviating the adverse effects of salinity (Fig. 7B).

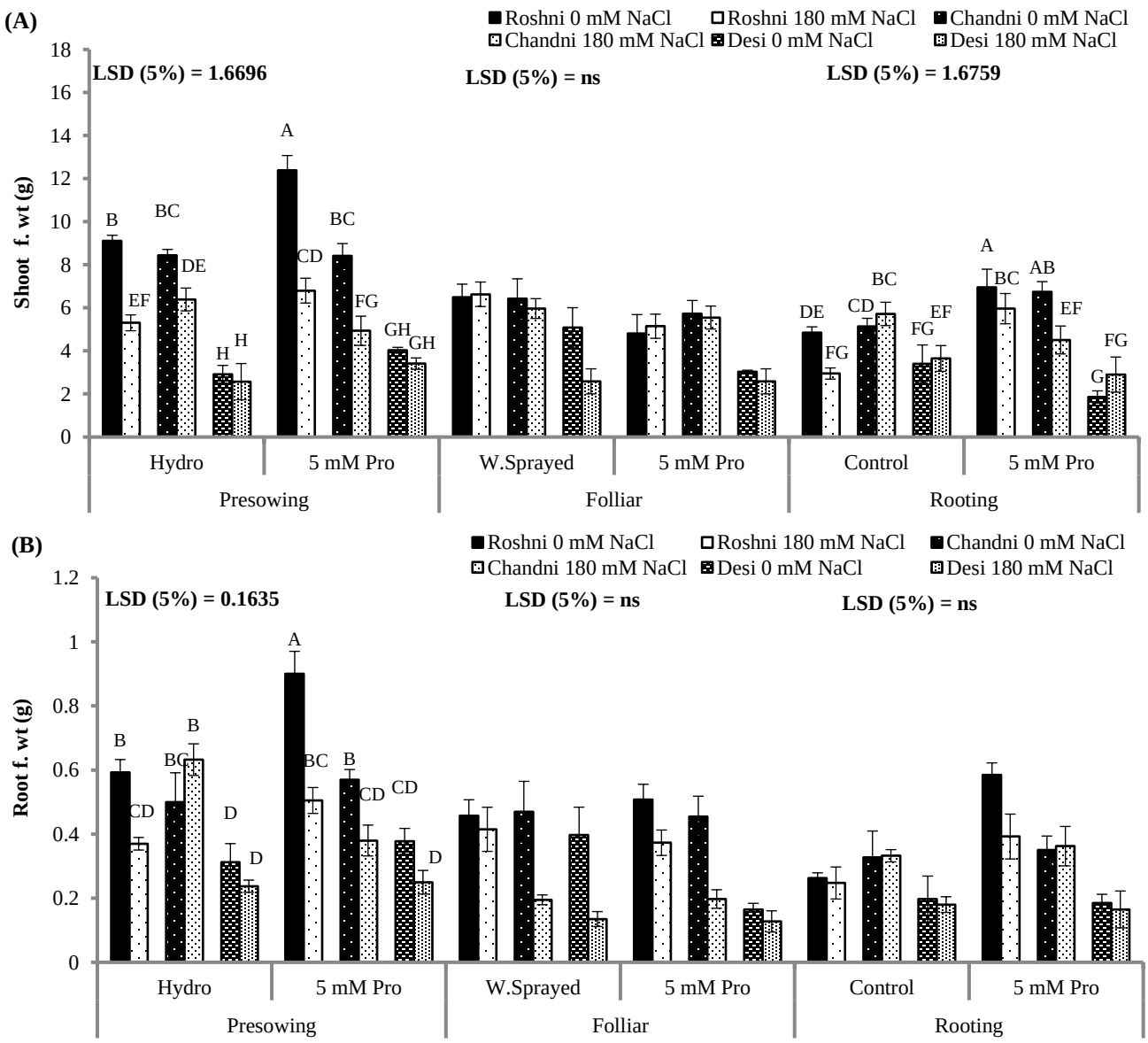

Fig. 1. Shoot (A) and root (B) fresh weights of the three flex genotypes applied with different modes of proline under saline conditions. Vertical lines on each bar represent ± SE values. Means sharing same alphabets are not-significant @ $p \geq 0.05$.

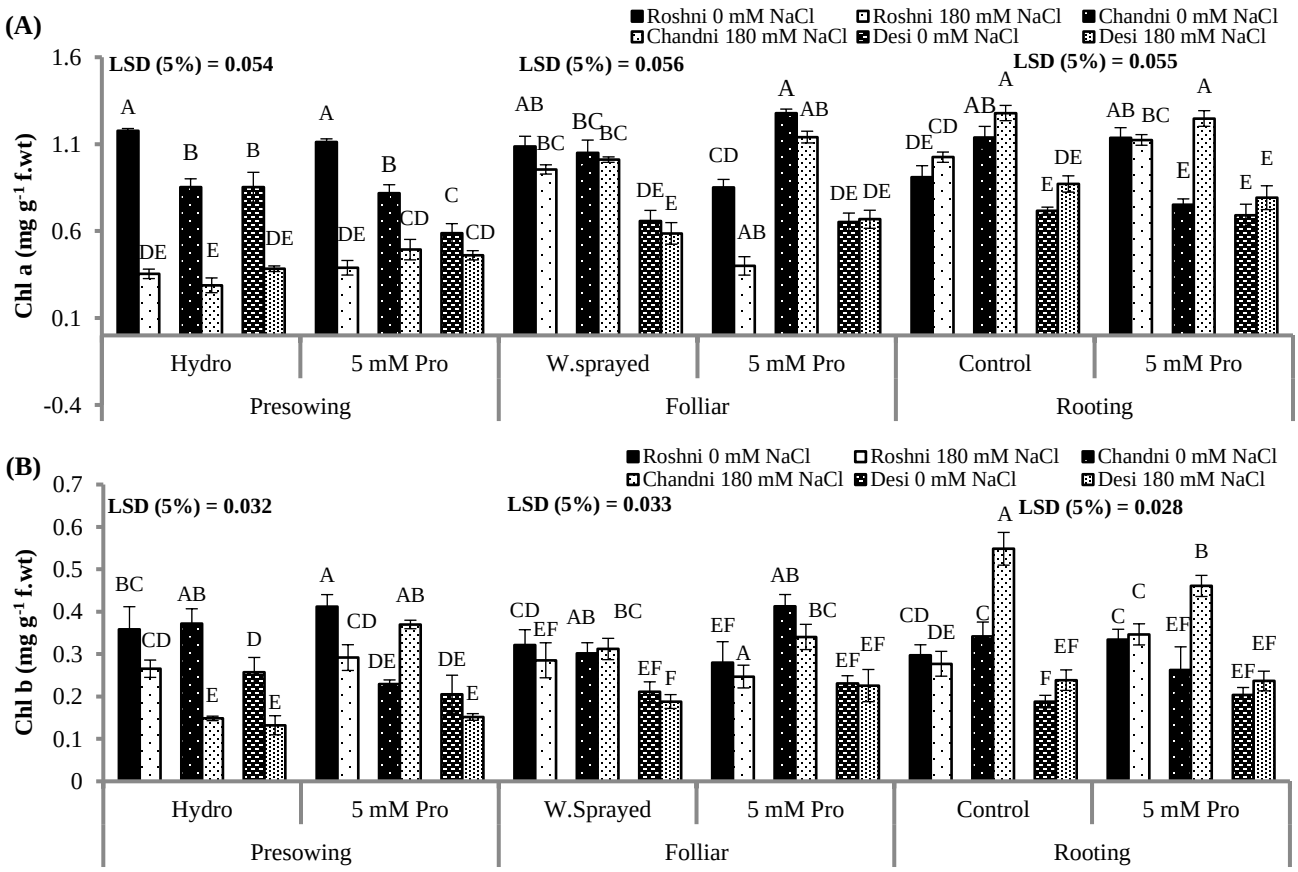

Fig. 2. Chlorophyll *a* (A) and *b* (B) of the three flex genotypes applied with different modes of proline under saline conditions. Vertical lines on each bar represent ± SE values. Means sharing same alphabets are not-significant @ p≥0.05.

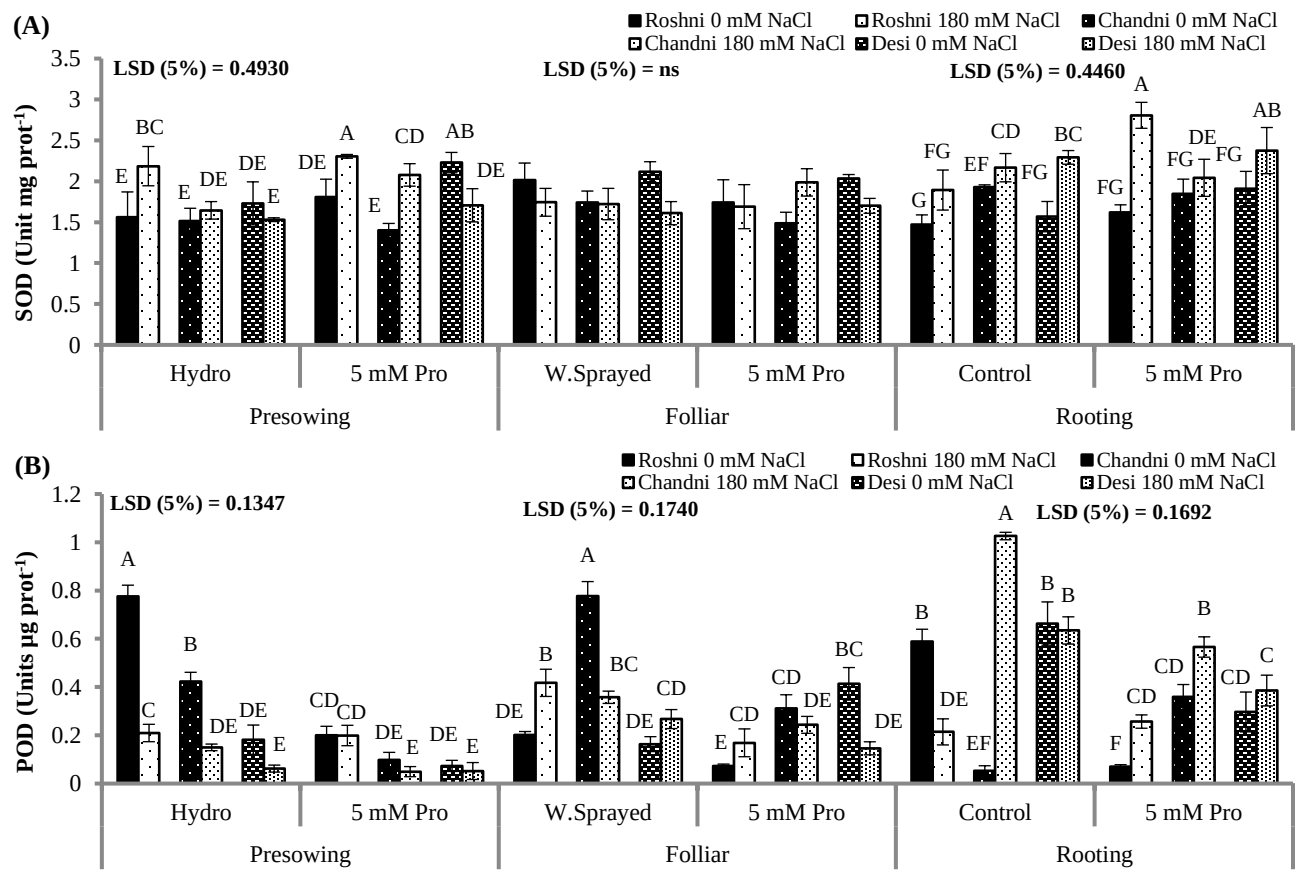

Fig. 3. SOD (A) and POD (B) activities of the three flex genotypes applied with different modes of proline under saline conditions. Vertical lines on each bar represent ± SE values. Means sharing same alphabets are not-significant @ p≥0.05.

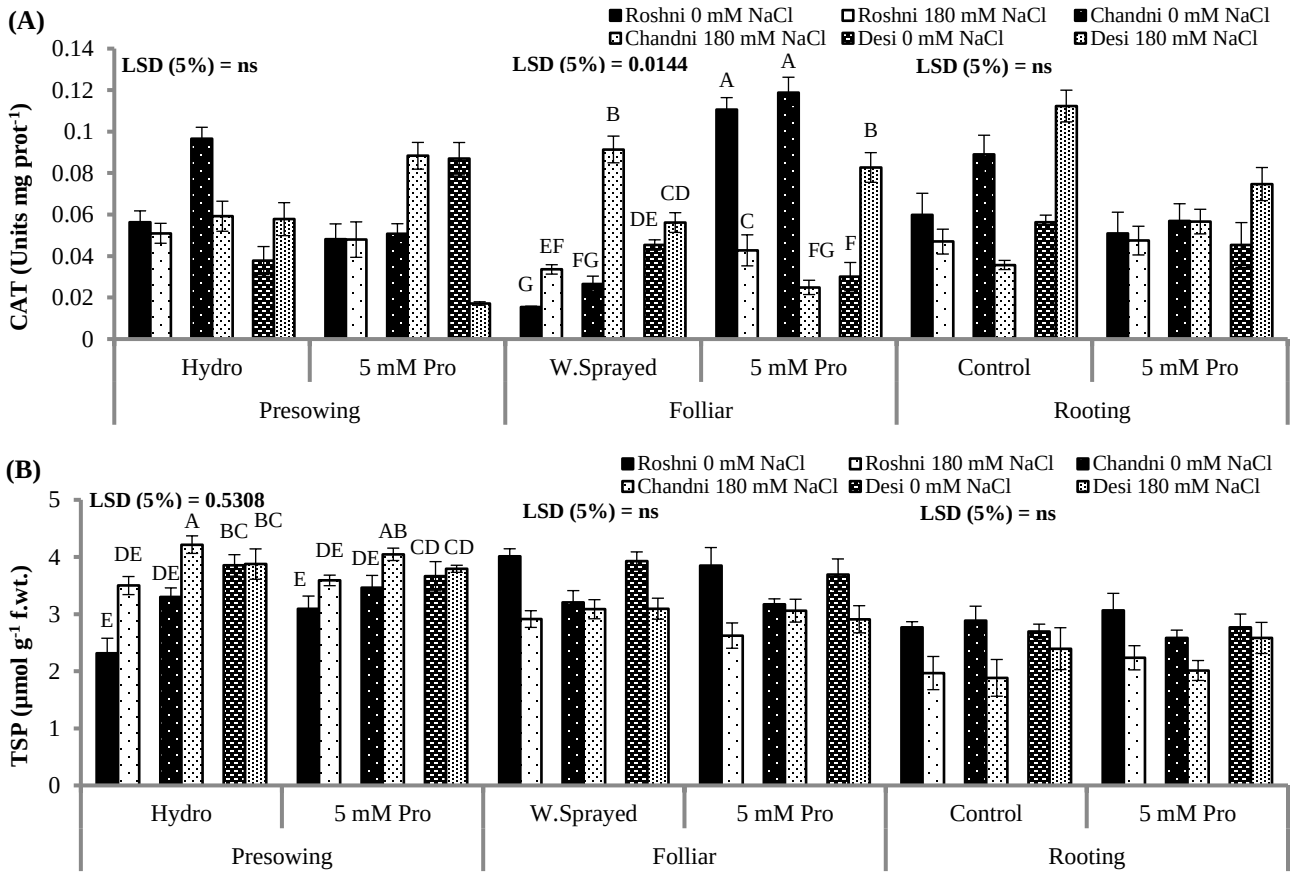

Fig. 4. CAT (A) and TSP (B) of the three flex genotypes applied with different modes of proline under saline conditions. Vertical lines on each bar represent ± SE values. Means sharing same alphabets are not-significant @ p≥0.05.

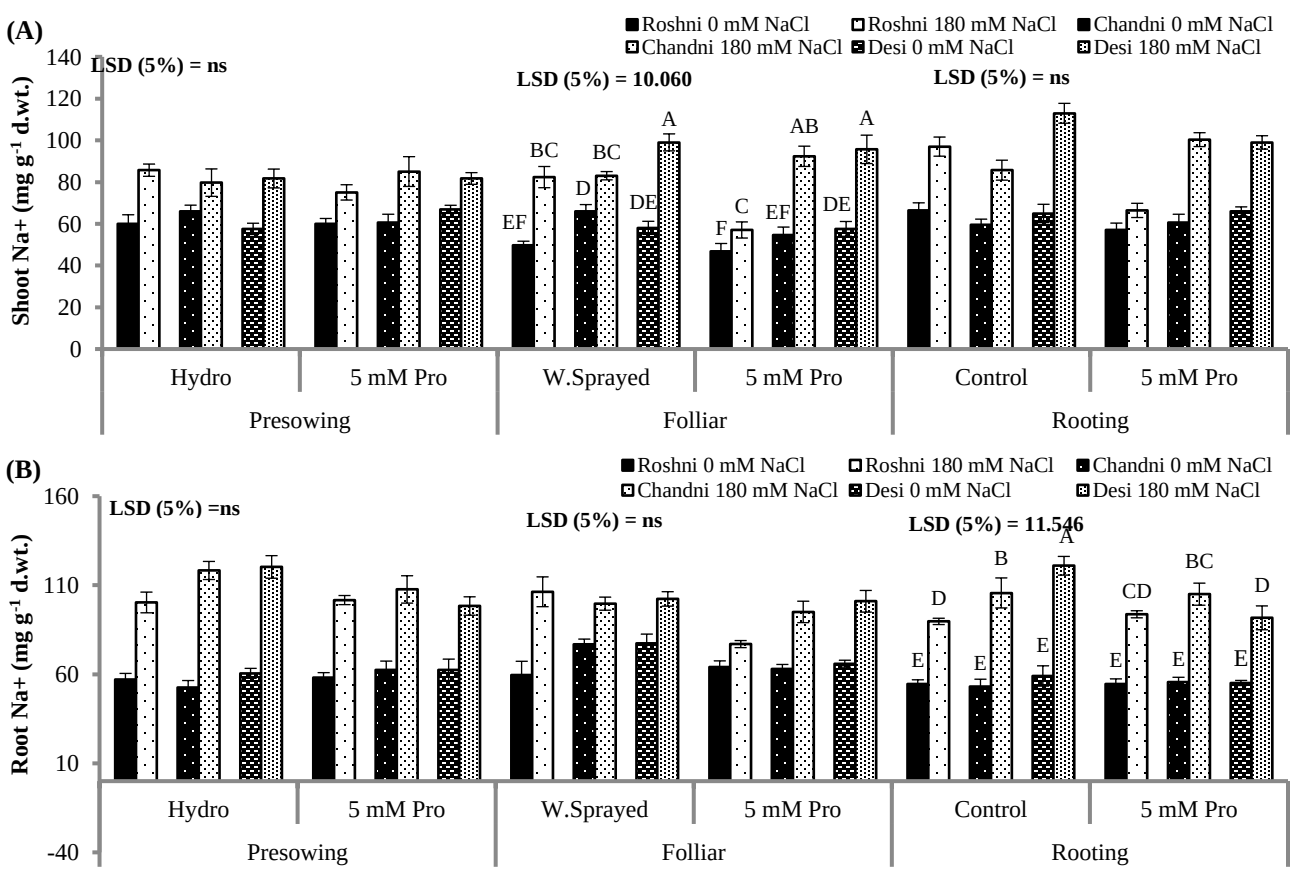

Fig. 5. Shoot (A) and root (B) Na^+ contents of the three flex genotypes applied with different modes of proline under saline conditions. Vertical lines on each bar represent ± SE values. Means sharing same alphabets are not-significant @ p≥0.05.

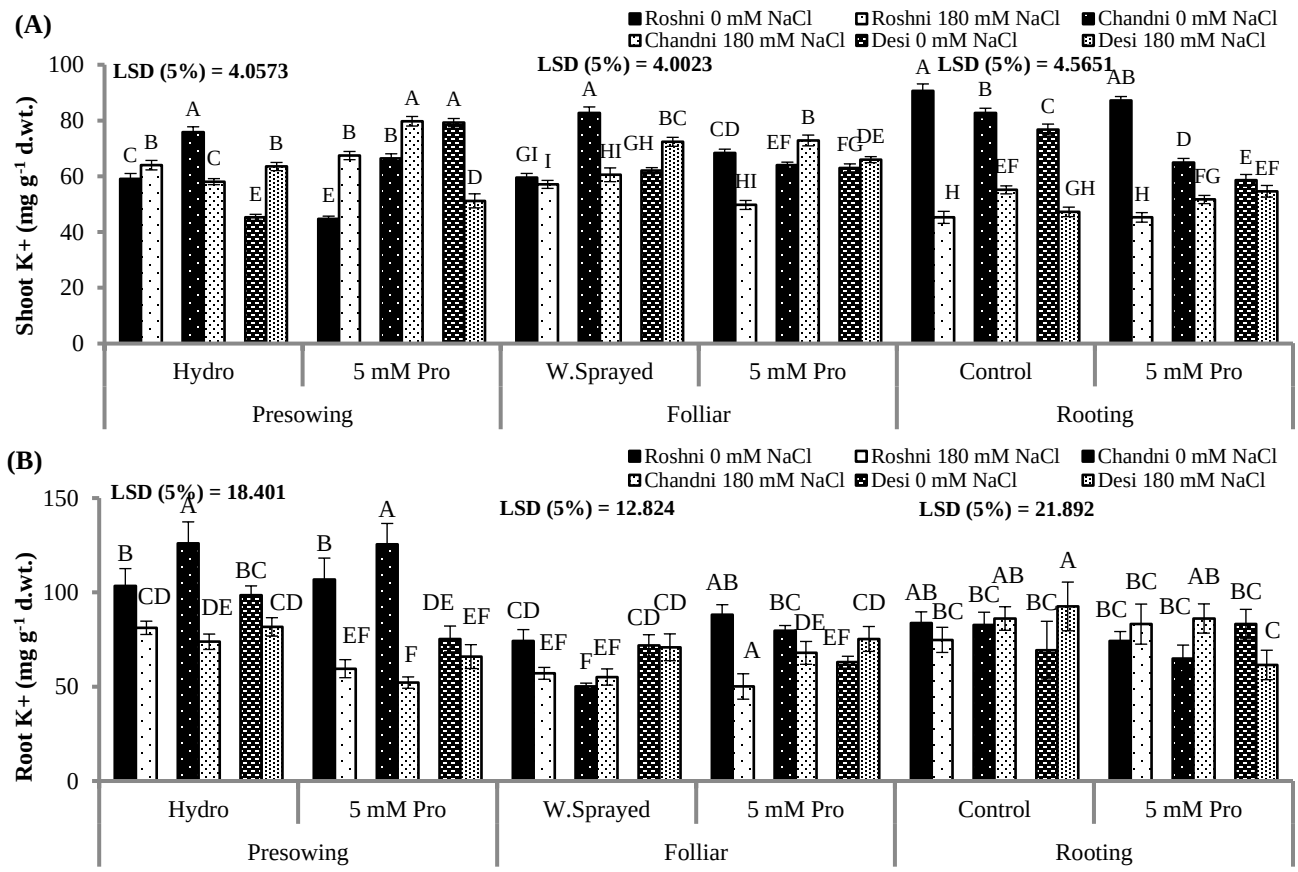

Fig. 6. Shoot (A) and root (B) K^+ contents of the three flex genotypes applied with different modes of proline under saline conditions. Vertical lines on each bar represent ± SE values. Means sharing same alphabets are not-significant @ p≥0.05.

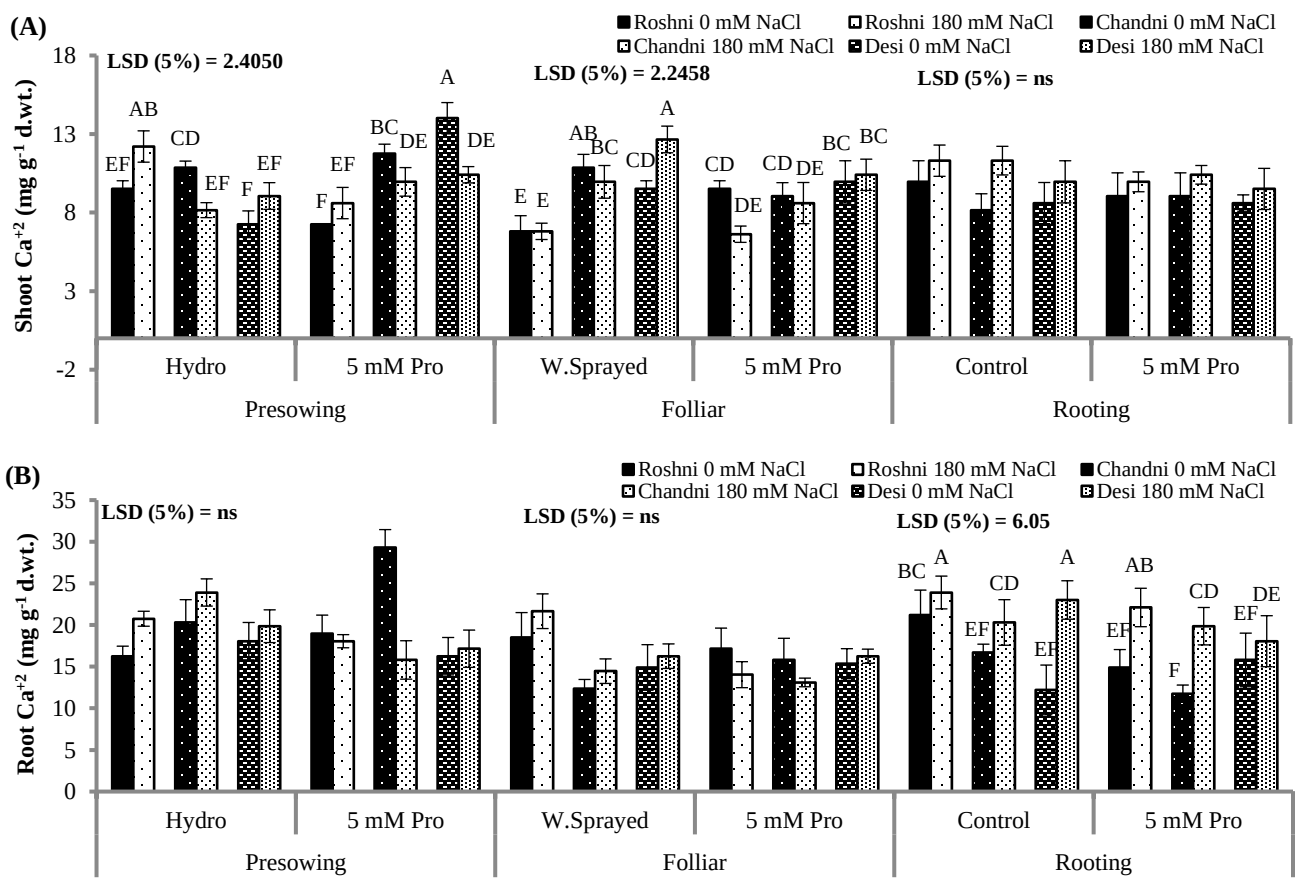

Fig. 7. Shoot (A) and root Ca^{2+} (B) of the three flex genotypes applied with different modes of proline under saline conditions. Vertical lines on each bar represent ± SE values. Means sharing same alphabets are not-significant @ p≥0.05.

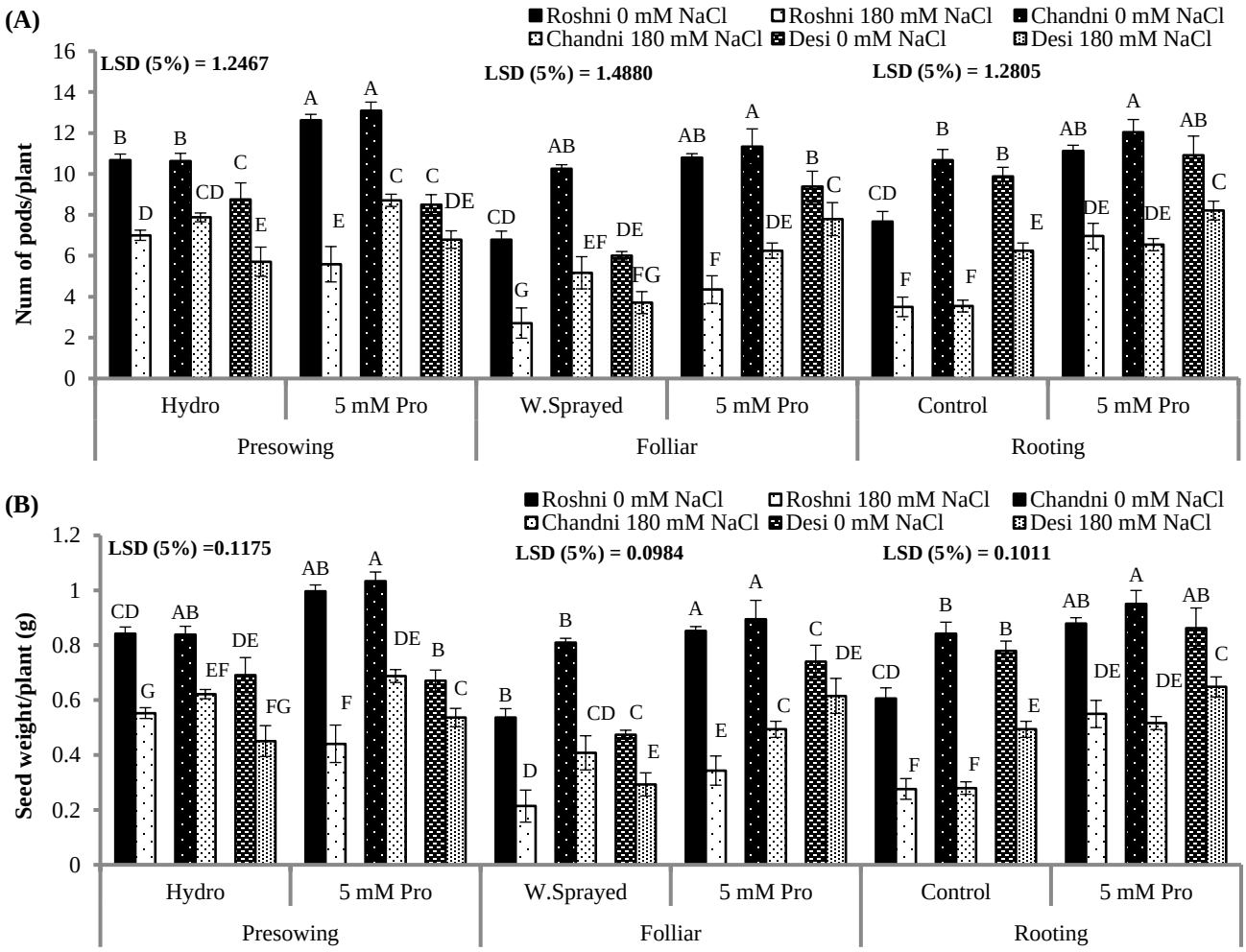

Fig. 8. Number of pods (A) and seed weight (B) per plant of the three flex genotypes applied with different modes of proline under saline conditions. Vertical lines on each bar represent ± SE values. Means sharing same alphabets are not-significant @ p≥0.05.

Yield parameters: The application of proline to flax genotypes under salinity stress had a significant impact on the number of pods per plant (Table 1, Fig. 8A). Proline application at 5 mM in all modes showed a significant (p≤0.05) effect on this attribute. While salinity significantly reduced flax yield, proline application improved yield parameters (Fig. 8A). The highest value was observed in the Chandni variety when 5 mM proline was applied in seed priming mode under non-saline conditions, whereas the lowest value was recorded in the Roshni variety under saline conditions. Proline application in all modes also had a significant (p≤0.05) impact on seed weight per plant (Table 1, Fig. 8B). While salinity reduced flax yield, proline application improved seed weight per plant. The highest value was observed in the Chandni variety when 5 mM proline was applied in seed priming mode under non-saline conditions, indicating a positive effect on seed weight.

Heatmap clustering: In the heatmap clustering for Roshni variety of flax, the first cluster containing SOD, RCa, SNa and RNa showed a strong positive relation with saline-control and saline-rooting mode of proline application. The 2nd cluster contained POD, CAT and SCa that showed positive relation with different modes. Here, POD showed positive relation with both hydro-primed and control, CAT with foliar, and, SCa with saline-control and saline-hydro-primed proline treatments. The 3rd cluster was the shortest one which contained only TSP and SK. Both these attributes showed strong positive relation with rooting, foliar, control and water-spraying proline treatments. The 4th one was the last and largest cluster which contain NPP, SWP, SFW, RFW, Cha, Chb and RK which showed strong positive relation with pre-sowing, hydro-primed and saline-foliar proline application modes. Nevertheless, all these attributes showed less strong relationship with rooting and foliar treatments (Fig. 9A).

The heatmap in Figure 10 showed the relationships between various morphological, biochemical, and growth attributes in the Chandni variety under different treatments. The heatmap revealed three distinct clusters: The first cluster showed a strong interconnection between SOD, SNa, and RNa with control and rooting, pre-sowing, and foliar treatments under saline conditions. The second cluster represented Cha, Chb, POD, and SCa, which were strongly positively connected to control and rooting under saline conditions. The third cluster, the largest one, comprised RK, RCa, NPP, SWP, SFW, and RFW, which were strongly positively linked to hydro-primed, pre-sowing, and saline-hydro-primed conditions, while CAT, TSP, and SK were positively connected to water-sprayed, foliar, saline pre-sowing, and hydro-primed proline treatments in both normal and saline conditions (Fig. 10A).

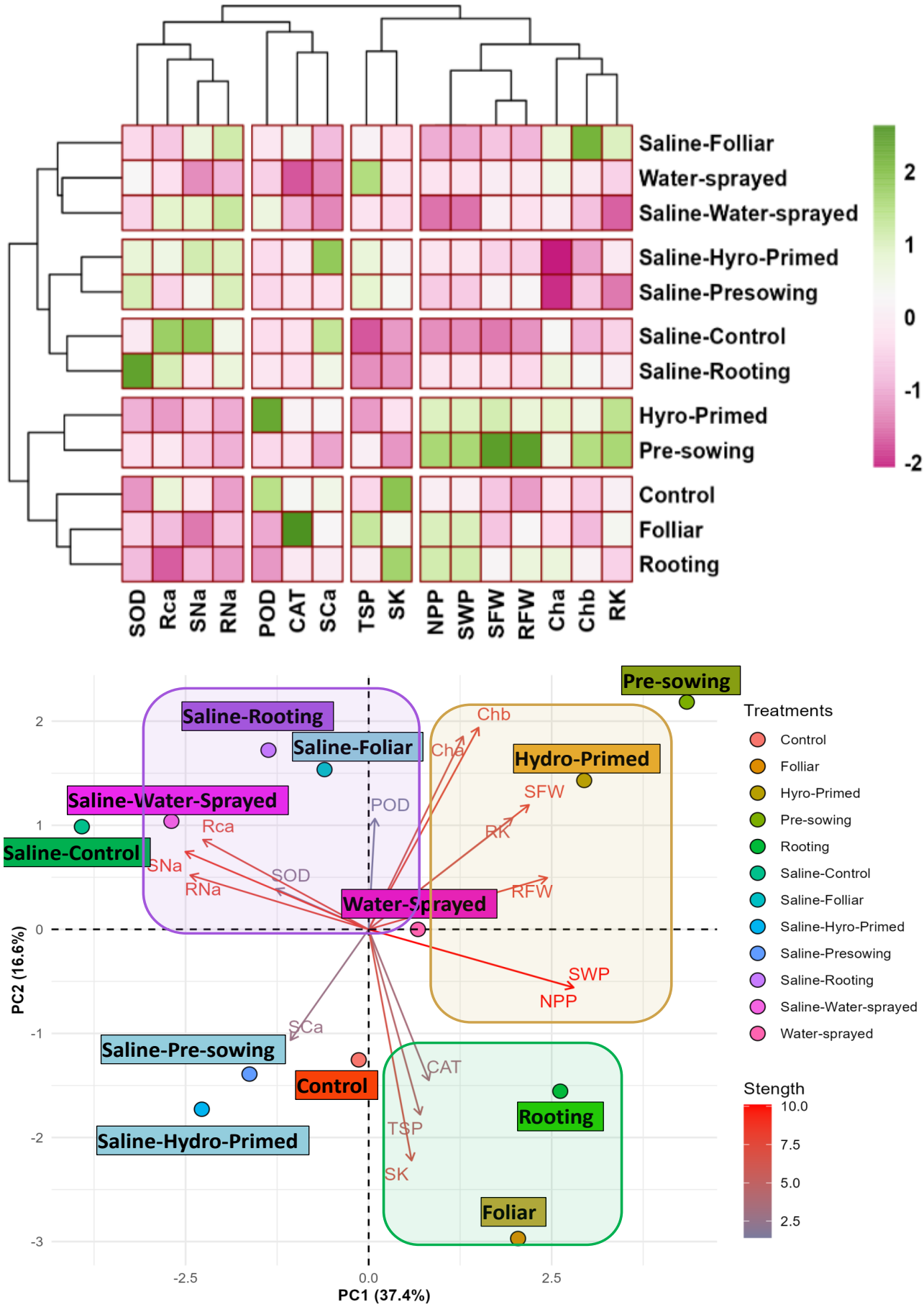

Fig. 9. Clustered heatmap and PCA showing grouping of various physiological, morphological, biochemical and yield attributes of flex genotype (Roshni) applied with different modes of proline under saline conditions.

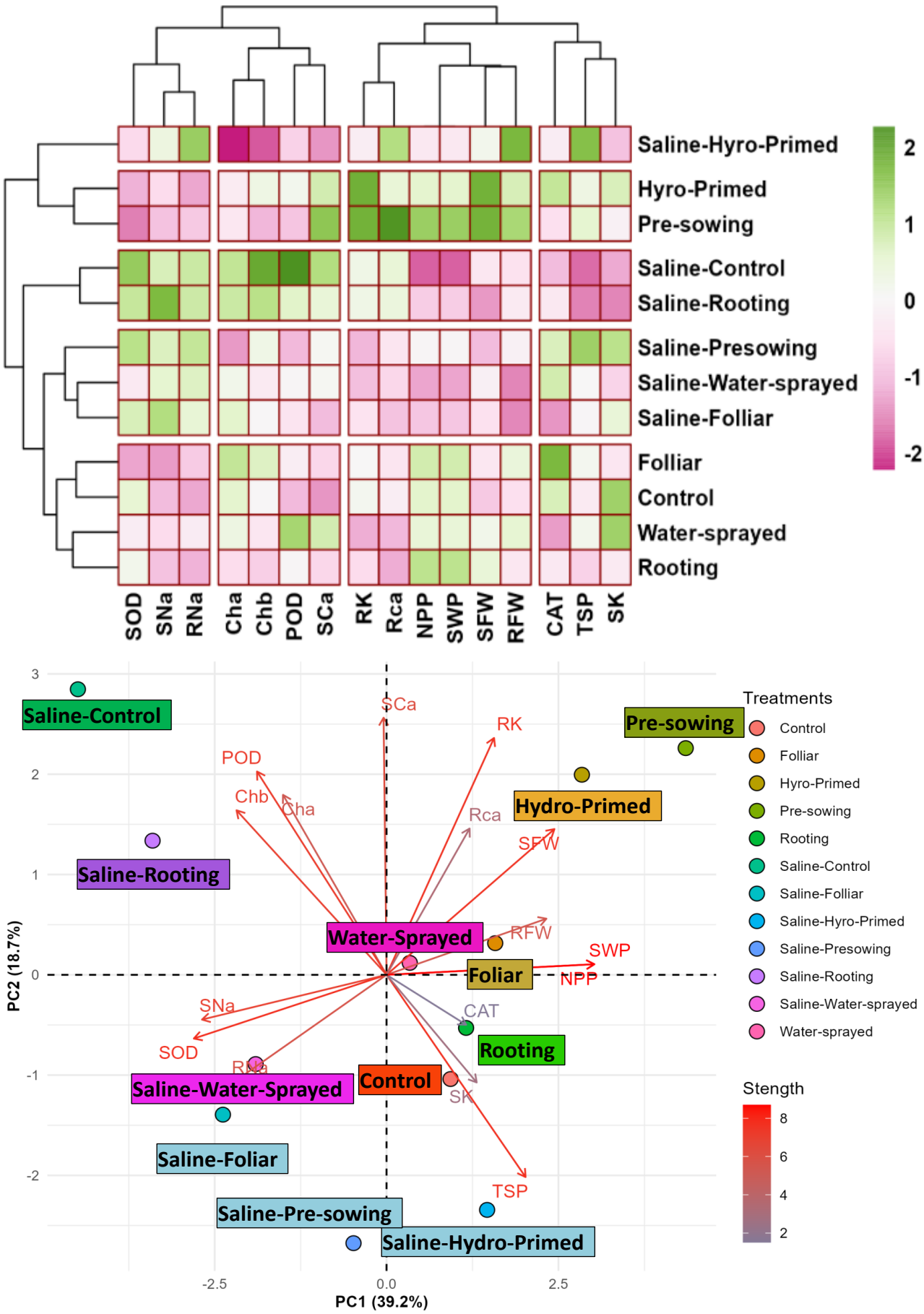

Fig. 10. Clustered heatmap and PCA analysis showing grouping of various physiological, morphological, biochemical and yield attributes of flex genotype (Chandni) applied with different modes of proline under saline conditions.

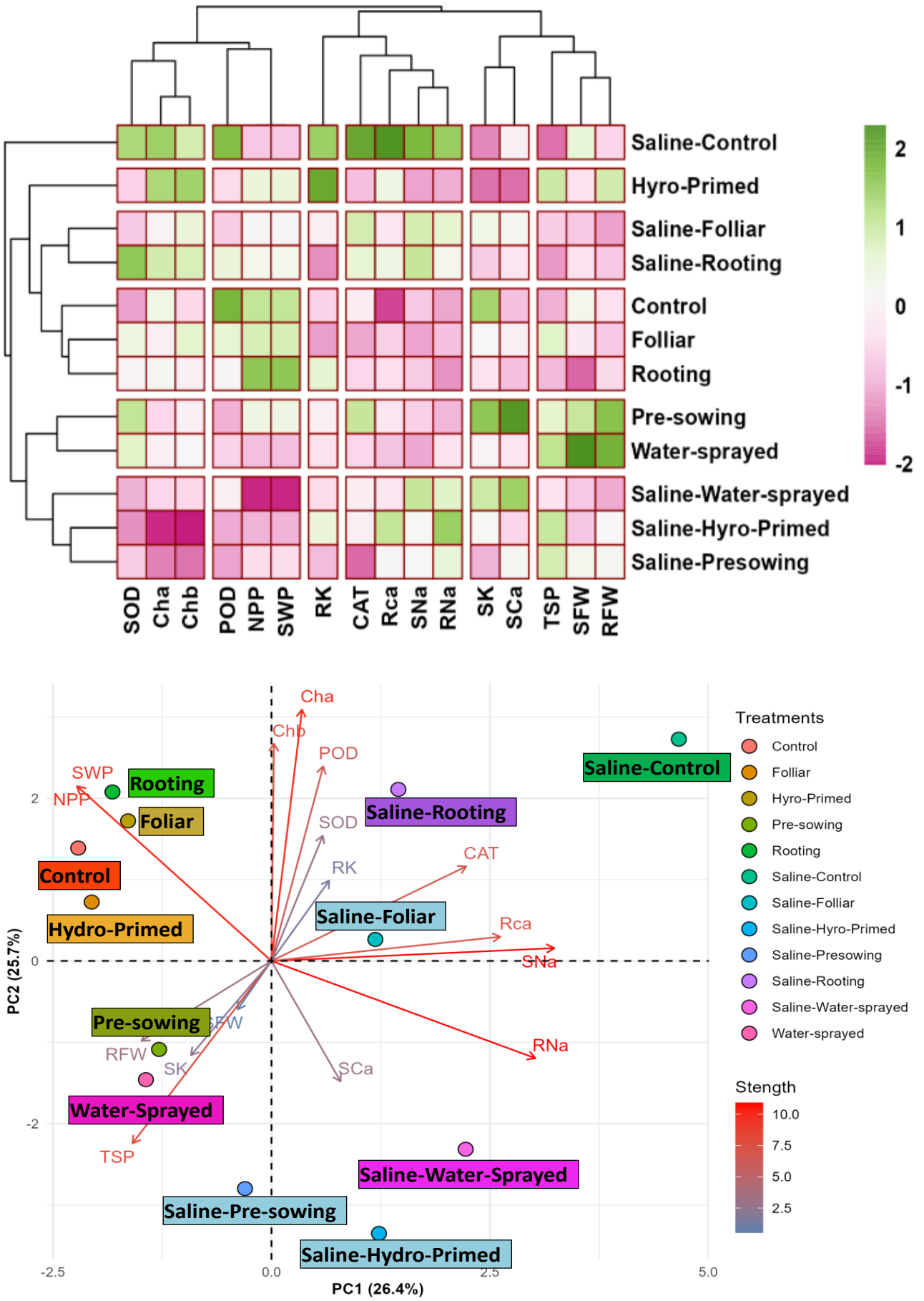

Fig. 11. Clustered heatmap and PCA analysis showing grouping of various physiological, morphological, biochemical and yield attributes of flex genotype (Desi) applied with different modes of proline under saline conditions.

The heatmap in Figure 11B illustrated the relationships between various morpho-physiological, biochemical, and growth attributes of the flax plant in the Desi genotype. The data was divided into quadrants, with four distinct clusters emerging therein. The first cluster consisted of SOD, Cha, and Chb, which were strongly linked to saline-rooting, hydro-primed, and saline-control conditions. The second cluster comprised POD, NPP, and SWP, which were strongly associated with rooting, control, and saline-control. The third cluster contained only RK, which had a strong positive relationship with hydro-primed and saline-control. The largest cluster, the fourth, included CAT, RCa, SCa, and RNa, which showed a strong interconnection with saline-control and were strongly linked to hydro-primed, rooting, and foliar under saline conditions. The next group contained SK and SCa, which had a strong positive connection with saline-water-sprayed, pre-sowing, and control. Finally, the last group included TSP, SFW, and RFW, which were positively linked to water-sprayed and pre-sowing proline treatments.

Principal component analysis (PCA): The PCA analysis presented in Figure 9B reveals the variation among different attributes in the Roshni variety of flax plant under various treatments. A total of 37.4% and 16.67% variation was explained by PC1 and PC2, respectively. The analysis shows that foliar and rooting applications of proline, water-sprayed, and control conditions under saline stress were associated with RNa, SNa, RCa, SOD, and POD. In contrast, hydro-primed conditions were strongly linked to Cha, Chb, RK, SFW, RFW, SWP, and NPP. Additionally, rooting and foliar modes of proline application showed a strong interconnection with CAT, TAP, and SK, while control, pre-sowing, and hydro-primed conditions under saline stress had a strong positive relationship with SCa.

The PCA analysis in Figure 10 for the Chandni genotype of flax plant revealed that 39.2% and 18.7% of the variation was explained by PC1 and PC2, respectively. The analysis showed that rooting and control treatments under saline conditions were strongly linked to Cha, Chb, POD, and SCa, while water-sprayed treatment was associated with these attributes as well as RK, RCa, SFW, RFW, and SWP. Hydro-primed condition had a strong positive correlation with RK, RCa, and SFW, and foliar treatment was linked to RFW, SWP, and NPP. Additionally, rooting was associated with CAT, control was correlated with SK. The hydro-primed treatment under saline conditions was positively linked to TSP, while foliar and water-sprayed conditions under saline environment showed a strong interconnection with RNa, SOD, and SNa.

The PCA analysis represented in Figure 11 for the Desi variety showed that PC1 and PC2 explained 26.4% and 25.7% of the variation, respectively. The analysis revealed that control, foliar, and rooting treatments were strong positively linked to NPP and SWP. Foliar and rooting proline treatments under saline conditions were associated with Cha, Chb, POD, SOD, RK, CAT, RCa, and SNa. In contrast, hydro-primed and water-sprayed conditions under saline conditions were strongly linked to SCa and RNa. Additionally, pre-sowing, water-sprayed, and saline-pre-sowing treatments had a strong positive link with TSP, SK, RFW, and SFW. This analysis highlighted the relationships between various attributes and treatments in the Desi variety.

Discussion

High levels of salinity as a global environmental factor can inhibit plant development and crop productivity (Yang & Guo, 2018). Plant growth is regarded an important criterion for salinity tolerance (Shahbaz *et al*., 2011). Seed germination, plant growth, development, and yield are all repressed by salinity stress (Zhang & Dai, 2019). In this study, the application of salinity inhibited root and shoot growth in the flax plant. In different crops, such as flax, salinity stress caused significant and progressive reductions in all growth traits, physiological parameters like water relation, photosynthetic pigments and K^+/Na^+ ratio (El-Afry *et al*., 2018). Growth may be reduced as a result of high Na and Cl ion uptake by plants, which alters the metabolic process (Ahanger & Agarwal, 2017). Imbalanced essential nutrient uptake under salinity may also contribute to reduced root and shoot growth (Jamil *et al*., 2006). Proline application under saline conditions increased plant growth and enhanced root and shoot dry weight and fresh in our study. Previously, similar results have been observed in *Brassica juncea* (Wani *et al*., 2016) and *O. sativa* (Teh *et al*., 2016).

Photosynthesis is the most important and significant biochemical processes that plants use to transform solar energy into chemical energy and to grow. Reduced water potential is the main cause of the decline in photosynthetic rates in plants under salt stress. Chlorophyll, a crucial element in photosynthesis and directly related to plant health, is inhibited when large concentrations of Na^+ and Cl build up in chloroplasts (Zhang *et al*., 2005). In a past study on *O. sativa*, chlorophyll a and b contents of leaves were reduced after NaCl treatment. The chlorophyll b content of leaves was more negatively impacted (41 percent) than the chlorophyll a content of leaves (33 percent) (Amirjani, 2011). In our work as well, salinity-exposed flax plants had higher levels of all photosynthetic pigments and carotenoids when imposed to exogenously supplied proline. The exogenously applied proline has positive impact on plant development under salinity stress that is connected to the changes in photosynthetic activity (Hayat *et al*., 2012; Mansour & Ali, 2017). Exogenous proline, according to Nawaz *et al*., (2010), increased the levels of chlorophyll a and total chlorophyll in salt-stressed *S. bicolor*. Chlorophyll b content did not significantly differ between 50 and 100 mM (NaCl). Those results strongly imply that exogenous proline influences plant development when exposed to salinity via enhancing photosynthesis.

All biological macromolecules in the cell are impacted by ROS because of their high reactivity. Reactive oxygen species include superoxide anion (O_2^-), hydroxyl radical (•OH), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) (Mittova *et al*., 2003). Plants oxidative defense mechanisms develop to protect them from impact of reactive oxygen species (ROS). In addition to the primary antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR), this system also includes non-enzymatic antioxidants such as glutathione, ascorbate, tocopherol, and different phenolic substances (Abbasi *et al*., 2014; Anjum *et al*., 2016). According to a number of publications, the ability of plants to tolerate salt

is accompanied by a concurrent increase in antioxidant enzyme activity when they are exposed to salinity (Hernandez *et al*., 2000; Rubio *et al*., 2009). According to a recent study in sugar beet (*Beta vulgaris*), POD and CAT activity increased and was regarded as an indicator of increased salinity tolerance in sugar beet (Tahjib-ul-Arif *et al*., 2019).

The K^+ ion makes up 10% of the dry biomass and is crucial for the development and growth of plants. Normal plant cell function requires substantial cytoplasmic Na^+ and K^+ ion concentrations. Salinity stress changes the K^+/Na^+ ratio, which causes an increase in the concentration of Na^+ (Shabala & Pottosin, 2014). Decreased cell homeostasis and oxidative stress follows from Na^+ displacement of K^+ from the cell (Cabot *et al*., 2014). It is well recognized that nutritional problems brought by salinity can negatively affect crop performance. Salinity lowers plant nutrient uptake and accumulation, according to a number of studies (Rogers *et al*., 2003; Hu & Schmidhalter, 2005). A high NaCl treatment increased the sodium (Na) concentration in the plant tissues while lowering the Ca^{2+}, K^+, and N levels in the leaves (Tuna *et al*., 2007). Overall nutritional deficit is caused by high salt content, which elevates Na^+ and Cl^- in plants while thereby lowering NO_3, S, Ca^{2+}, K^+, Mg^{2+} and other vital minerals (Manchanda & Garg, 2008; Farissi *et al*., 2014). Proline has been shown to improve plant tolerance to salinity and promote nutrient absorption in numerous experiments. Application of exogenous proline decreased the Na^+ while increased the potassium and calcium ion in both root and shoot of flax plant in current investigation. At various salinity levels, exogenous proline application enhanced the NO_3, NO_2, K, and P concentrations in *Phaseolus vulgaris* (Abdelhamid *et al*., 2013). Proline increased Ca^{2+} and K^+ contents in *Sorghum bicolor* under salty conditions as well as (de Freitas *et al*., 2019) in *O. europaea* (Ben Ahmed *et al*., 2011). According to Alam *et al*., (2016), exogenous proline may improve P, N, K^+, and S absorption in *Z. mays* in saline conditions.

The most detectable impact of salinity in agriculture is the decline in crop production that results from all above mentioned impacts of salinity stress on plants. Salinity stress had a considerable effect on different yield components, as reported in review. Salinity levels were negatively linked with number of seeds per pod, seed weight and number of pods per plant (Hasanuzzaman *et al*., 2009). Reduction in grain productivity of rice variety caused by the high salinity was reported by Linghe & Shannon (2000) and Gain *et al*., (2004). According to a recent study, proline treatments had a positive impact on the quality and yield of the wheat cultivar grown in sandy soil (Dawood, 2021). Proline, on the other hand, functions as a compatible osmolyte with the remarkable capacity to stabilize the cellular redox potential, protect enzymes, and improve membrane stability. Tolerance to salt is positively correlated with its higher concentration of osmolyte (El-Bassiouny & Sadak, 2015; Sadak *et al*., 2020).

Conclusions

Negative effects of salinity stress on growth, photosynthetic activity and yield of flex plants were observed. The exogenous application of 5 mM proline at 0 and 180 mM salinity levels reduced oxidative stress and improved the length, fresh weight and dry weight of flax plants. The addition of proline also raised the concentration of carotenoids, chlorophyll levels, Ca^{2+} and K^+ and activity of anti-oxidative enzymes (SOD, POD and CAT). Applying proline to salt stressed plants also resulted in an increase in Ca^{2+} and K^+ concentrations and decrease in Na levels. It was concluded that 5mM proline concentration showed the greatest enhancement in growth and yield of salt stressed flax plants. As shown by PCA model, application of proline to the leaves (foliar application) was the most effective method for mitigating the harmful effects of salinity stress, as compared with rooting application or water spray.

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