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Full Length Article

Soil Biochar Ameliorates Salinity Stress and Improves Nutrient Uptake, Biomass Production and Physiochemical Parameters in Sunflower

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Abstract

A pot experiment was set-up to assess the modulations in growth, oxidative defense, osmotic adjustment, secondary metabolism, and ion homeostasis in sunflower (*Helianthus annuus* L.) sown in corncob biochar amended (0, 1, 2 and 3%) soil under salinity stress. Sunflower cultivars (FH-687 and FH-697) were subjected to NaCl salinity (0 and 15 dS m^{-1}) three weeks after germination. Salinity resulted in significant decrease in growth, photosynthetic pigments, RWC, K^+, P contents and increase in Na^+, Ca^{2+}, MDA, RMP, H_2O_2, TFAA, TSP, TSS, GB, and proline. Biochar (3%) enhanced the activities of antioxidant enzymes and concentration of non-enzymatic antioxidant compounds (flavonoids, phenolics, and ascorbic acid). Biochar increased the cellular concentrations of glycine betaine and free proline that resulted in better osmotic adjustment under saline conditions. Biochar-induced upregulation of oxidative defense and decrease in specific ion toxicity was variable in two cultivars. Cultivar FH-687 exhibited better salinity tolerance compared with FH-697 in the form of greater oxidative defense and osmotic adjustment alongside the lower specific ion toxicity.

Keywords: Salt sorption; Salt stress; Non-enzymatic antioxidants; Oxidative damage; Ion homeostasis; Osmotic adjustment
Abbreviations: RWC, leaf relative water content; K^+, potassium; P, phosphorous; Na^+, sodium; Ca^{2+}, calcium; MDA, malondialdehyde; RMP, relative membrane permeability; H_2O_2, hydrogen peroxide; TFAA, total free amino acids; TSS, total soluble protein; GB, glycine betaine

Introduction

Soil salinity in several arid and semi-arid regions of the world arises due to poor agricultural practices and natural sources (Farhangi-Abriz and Torabian, 2018). Salinity causes nutritional imbalances, specific ion toxicity, osmotic stress, and oxidative damage that impair plant growth and productivity. Salinity tolerance may vary with plant species and developmental stages reflected in the form of diminished growth, productivity, and rate of survival. Salinity affects metabolism and all growth stages in plants. In this context, reduced seed germination is the first critical event followed by retarded plant growth and productivity (Iqbal *et al.*, 2018).

Physiological drought seriously influences plant morphology and physiology. It is created due to salinity-induced osmotic stress that impairs nutrient and water uptake leading to the dysfunction of all metabolic events. The reactive oxygen species (ROS; O_1, H_2O_2, OH^- and $O_2^{\cdot-}$) further aggravate the metabolic failure. ROS causes oxidative damage in the form of lipid peroxidation, chlorophyll degradation, and damage to DNA, lipids, and proteins (Hasanuzzaman *et al.*, 2018). The cellular buildup of malondialdeyde (MDA), a by-product of lipid peroxidation, is a crucial yardstick to assess plant salt tolerance (Rasheed *et al.*, 2018). To protect different cellular compartments from ROS-induced oxidative stress, plants possess defense mechanism in the form of antioxidant enzymes and non-enzymatic antioxidants (Iqbal *et al.*, 2018). NaCl is the major component in soil salinity. Plants under salinity show buildup of toxic ions (Na^+ and Cl^-) that hamper normal cell functions. Furthermore, Na^+ and Cl^- disturb nutrient uptake either through changing membrane selectivity or competitive interaction (Farhangi-Abriz and Torabian, 2018). Higher Na^+ levels in soil decrease potassium (K), magnesium (Mg), and calcium (Ca) uptake (Tester and Davenport, 2003). Changes in osmotic balance occur because of replacement of K ions by Na^+ under saline conditions (Iqbal *et al.*, 2018).

Biochar is a carbon-rich substance produced as a result of pyrolysis of organic materials. Biochar can be obtained by heating organic waste of any nature such as plant residues in

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a process referred to as pyrolysis (Farhangi-Abriz and Torabian, 2018; Kanwal *et al.*, 2018). Biochar influences soil physical (e.g. bulk density, aeration, aggregate stability, and soil moisture-holding capacity), chemical (e.g. CEC, pH, EC, and nutrient holding capacity) and biological (e.g. enzymatic activities, microbial biomass N and C, and rhizosphere microbial population) characteristics (Akhtar *et al.*, 2015; Riaz *et al.*, 2018; Zhang *et al.*, 2018). The high salt adsorption ability of activated charcoal has long been known. Likewise, biochar also exhibits salt adsorption capacity and could be applied to circumvent salinity (Akhtar *et al.*, 2015; Kanwal *et al.*, 2018). However, limited information is available in the literature on the use of biochar to ameliorate salinity in plants. The reported biochar effects may vary with plant species.

Sunflower (*Helianthus annus* L.) serves as a rich source of edible oil for the world (Talia *et al.*, 2011). However, soil salinization is the major obstacle for sunflower production since sunflower is more sensitive to salinity compared with cereal crops (Habibi, 2017). Despite the previous reports, there is limited information on biochar-mediated salinity tolerance in sunflower. Therefore, our objectives were to evaluate the hypothesis that soil biochar amendment may increase nutrient availability and oxidative defense system to circumvent salinity stress. Furthermore, our hypothesis also involved examining whether or not toxic Na^+ uptake is reduced as a result of biochar Na^+ adsorption capacity. We examined how far osmotic adjustment of salinity stressed plants is improved due to biochar addition. The ameliorative effect of biochar on plant mineral nutrition under salinity was also studied.

Materials and Methods

Seeds of two sunflower cultivars namely (FH-687 and FH-697) were surface sterilized with 10% H_2O_2 and thoroughly rinsed with distilled water. Five seeds of each sunflower cultivar were sown in plastic pots filled with 10 kg of loamy clay soil. Each pot was fertilized with K_2O, P_2O and N (60:75:120 kg ha^{-1}) in the form of muriate of potash (60% K_2O), single super phosphate (18% P_2O_5) and urea (46% N), respectively (Saeed *et al.*, 2016). Biochar from corncob was prepared with the help of procedure given by Sanchez *et al.* (2009). Biochar production was carried out according to the procedure described by Riaz *et al.* (2017). Different levels of biochar (0, 1, 2 and 3% w:w) were achieved in soil before sowing. The physicochemical properties of soil and biochar used in the present experiment are given in Table 1. Two weeks after germination, thinning was performed to keep two plants in each pot. Three weeks after germination salinity treatments were started by adding NaCl to achieve 15 dS m^{-1} EC. The procedure given by Habibi (2017) was followed to apply salinity in the soil. Briefly, NaCl was dissolved in distilled water at rates of 0.2–1 g and applied to the soil. EC of the saturated soil paste was evaluated after 24 h. Afterward, the quantity of NaCl was estimated with the help of regression equation to attain desired salinity in soil (15 dS m^{-1}). The EC of control plants was 1.34 dS m^{-1}. The experiment was performed under natural climatic conditions where mean relative humidity was 30.15±2.78; day and night temperature 30.95±4.15 and 17.85±6.75°C, respectively. Plants were harvested three weeks after the application of salinity treatment and data for different growth, and biochemical attributes were obtained.

Growth Attributes

Plant samples were carefully uprooted and rinsed thoroughly with distilled water. Samples were then separated into root and shoot. Blotting paper was used to dry out the plant material before getting fresh weight. For dry weight plant samples were put in an oven at 70°C for one week.

Photosynthetic Pigment Analysis

Chlorophyll contents were extracted from fresh leaf tissue (0.1g) in the dark with the help of aqueous acetone (80%) at 4°C by inconsistent shaking until the leaves appear colorless. The absorbance of the supernatant was read at 480, 645, and 663 nm (Arnon, 1949).

Relative Water Content (LRWC)

Fresh weight of young leaf recorded, and leaf was immersed in distilled water for 3 h at 25°C. Then the leaf was removed from water and blotted dry with tissue paper for measuring the turgid weight. Afterward, the leaf was oven-dried for 15 h at 80°C. The measurements of fresh, dry and turgid weights were used in the following formula to calculate LRWC (Jones and Turner, 1978).

$$\text{RWC} = \frac{\text{Fresh wt} - \text{dry wt}}{\text{Turgid wt} - \text{dry wt}} \times 100$$

Osmolyte Analysis

Total soluble sugars (TSS): Fresh leaf tissue (0.25 g) was extracted in 5 mL of 80% aqueous ethanol. Sample extract (100µL) was reacted with 3 mL anthrone reagent (prepared in 72% sulfuric acid). The mixture was put in water bath at 95°C for 10 min. Then the mixture was cooled down at 25°C, and absorbance was read at 625 nm (Yemm and Willis, 1954).

Reducing sugars (RS): One mL of ethanol extract was reacted with 5 mL of *O*-toluidine (6%). The reaction mixture was heated at 95°C in a water bath for 20 min. Then the mixture was cooled down on ice, and the absorbance was read at 630 nm (Nelson, 1944).

Non-reducing sugars (NRS): The amount of NRS was calculated with the help of the formula given by Loomis and Shull (1937).

$$NRS = (total\ soluble\ sugars - reducing\ sugars) \times 0.95$$

Total free amino acids (TFAA): Fresh leaf material (0.5 g) was homogenized in 10 mL of 50 mM chilled potassium phosphate buffer (pH 7.5). The homogenate was centrifuged at 10000 × *g* at 4°C for 20 min. One mL of supernatant was added to the equal volume of acid ninhydrin and pyridine (10%) in the test tube. The reaction mixture was heated at 95°C in the water bath for 30 min. After that, the mixture was allowed to cool down at 25°C and volume was made to 7.5 mL with distilled water. The absorbance of the mixture was read at 570nm (Hamilton and Van Slyke, 1943).

Leaf free proline: Fresh leaf (0.25 g) was grinded in 5 mL of 3% sulfosalicylic acid and the homogenate was filtered. One mL of filtrate was reacted with acid ninhydrin and glacial acetic acid (1 mL of each). The mixture was heated at 95°C for 1 h. After that, toluene was added to the mixture when the mixture reached room temperature. Then test tubes were vortexed, and absorbance read at 520 nm (Bates *et al.*, 1973).

Glycine betaine (GB): Leaf material (0.25 g dry) was grinded to powdered form and mixed in 5 mL distilled water. The mixture was filtered and 1 mL of the filtrate was added to the test tube already having 200 µL of KI_3 and 1 mL of 2N H_2SO_4. The volume of the mixture was made to 5 mL with distilled water. The mixture was incubated at 4°C for 1 h and then 6 mL of 1, 2 dichloroethane added. Two layers appeared and absorbance of the lower layer was read at 365 nm (Grieve and Grattan, 1983).

Total Soluble Protein (TSP)

Sample (100 µL) was mixed with 2 mL Bradford reagent in the test tube. The mixture was kept at 2°C for 30 min. and absorbance read at 595 nm (Bradford, 1976).

Membrane Permeability, Oxidative Damage and Antioxidants

Relative membrane permeability (RMP): Fresh leaf tissue (0.5g) was cut to uniformly sized discs and put in the test tube with 10 mL deionized water. Test tubes were incubated at 25°C for 2 h, and EC_0 measured. Then test tubes were left overnight and EC_1 measured. Afterward, test tubes were autoclaved at 100°C for 1 h and EC_2 measured. The calculation was done with the help of following formula (Yang *et al.*, 1996).

$$\text{RMP} = \frac{(\text{EC1} - \text{EC0})}{(\text{EC2} - \text{EC0})} \times 100$$

Hydrogen peroxide (H_2O_2) analysis: Fresh leaf tissue (0.25 g) was homogenized with 5 mL 0.1% chilled TCA. The homogenate was centrifuged at 1000 rpm for 10 min. The supernatant was reacted with 500 µL of chilled 50 mM potassium phosphate buffer (pH 7.5) and 1 mL of KI. The samples were incubated for half an hour at room temperature before taking absorbance at 390 nm (Velikova *et al.*, 2000).

Table 1: Physico-chemical properties of soil used in the experiment

Soil Physico – chemical parameter	Value
pH	8.00
EC (d Sm^{-1})	1.34
Sand (%)	56.32
Silt (%)	37.13
Clay (%)	6.55
Textural Class	Sandy loam
Total organic carbon (%)	0.92
Total nitrogen (%)	0.05
C/N ratio	18.4
Physico-chemical characteristics of biochar	
$pH_{1:10}$	5.88
$EC_{1:10}$ (d Sm^{-1})	6.91
Total organic carbon (%)	73.27
Total nitrogen (%)	1.03
C/N ratio	71.14

Malondialdehyde (MDA) analysis: Fresh leaf tissue (0.25 g) was grinded in 5 mL of 5% TCA and centrifuged to get supernatant. Then 0.5 mL supernatant was reacted with 2 mL 0.5% TBA (thiobarbituric acid). The mixture was heated at 95°C for 50 min, cooled down at 25°C and absorbance read at 532 and 600 nm (Heath and Packer, 1968).

Non-enzymatic antioxidants: To determine the flavonoids, 1 mL of ethanol extract (the one used for TSS) was added to 300 µL $NaNO_2$ in the test tube and left for 5 min. at 25°C. Then 300 µL $AlCl_3$ was added to the mixture and again given incubation for 5 min. After that, 2 mL NaOH (1 M) was added and volume of the mixture raised to 10 mL with distilled H_2O. After 10 min. incubation at room temperature, the absorbance was read at 510 nm (Marinova *et al.*, 2005).

Phenolics were measured from fresh leaf tissue (0.25 g) grinded in 5 mL 80% aqueous acetone. After centrifugation, 100 µL supernatant was added in the test tube containing 1 mL of folin-ciocalteu phenol reagent and 2 mL distilled water. The test tubes were vortexed and added 5 mL 20% Na_2CO_3. The volume was made to 10 mL with distilled H_2O, and absorbance read at 750 nm (Julkunen-Tiitto, 1985).

Anthocyanins were measured from fresh leaf material (0.2 g) extracted in 1 mL of 1% HCL in methanol (v/v) and the homogenate was centrifuged at 13000 rpm for 20 min. The absorbance of the supernatant was read at 530 and 657 nm. The relative concentration of anthocyanin was defined as the product of extraction solution volume and anthocyanin concentration. One unit of anthocyanin is equal to one absorbance unit [A530 2 (1/4 3 A657)] in 1 mL of extraction solution. Following formula was used to calculate anthocyanin levels (Mita *et al.*, 1997).

For ascorbic acid analysis, fresh leaf sample (0.25 g) was grinded in 6% TCA and the homogenate filtered. Two mL of filtrate was reacted with 1 mL of 2% 2,4 dinitrophenyl hydrazine. Then one drop of 10% thiourea was added to the mixture. The samples were heated at 95°C for 20 min. and then allowed to cool down at room

temperature. After that, 2.5 mL of 80% H_2SO_4 was added to mixture on ice and absorbance read at 530 nm (Mukherjee and Choudhuri, 1983).

Enzymatic antioxidants assay: Giannopolitis and Ries (1977) method was followed to determine SOD activity from potassium phosphate buffer extract (the one used for TFAA and TSP). The reaction volume (3 mL) consisted of 75 nM EDTA, 1.3 µM riboflavin, 13 mM methionine, 50 mM potassium phosphate buffer (pH 7.5), 50 µM NBT and 50 µM enzyme extract. The tubes without enzyme extract served as control. After that, tubes were illuminated with the 30 W lamp for 10 min. The absorbance of reaction mixture was read at 560 nm. One unit of SOD was considered as the amount of enzyme needed to cause 50% inhibition in photochemical reduction of NBT.

The CAT activity was assayed with the help of Chance and Maehly (1955) method. The reaction solution contained 1 mL H_2O_2 (5.9 mM), potassium phosphate buffer (50 mM; pH 7.5) and 100 µL enzyme extract. The absorbance of reaction mixture was read at 240 nm after every 20 seconds for 2 min. Absorbance change of 0.01 per min. was defined as one unit of CAT activity.

For POD activity, the reaction volume (3 mL) consisted of potassium phosphate buffer (50 mM; pH 7.5), guaiacol (20 mM) and H_2O_2 (5.9 mM) and 100 µL enzyme extract. Change in absorbance was read at 470 nm after every 20 seconds for 2 min. Absorbance change of 0.01 per min. was defined as one unit of POD (Chance and Maehly, 1955).

For APX activity, the reaction volume for APX (1 mL) contained potassium phosphate buffer (50 mM; pH 7.5), ascorbic acid (0.5 mM), H_2O_2 (0.1 mM) and 200 µL enzyme extract. Change in absorbance was read at 290 nm after every 20 seconds for 2 min. The enzyme activity was expressed in units mg^{-1} protein (Nakano and Asada, 1981).

Ionic and Nutrient Analysis

Digestion of plant material: Two mL of concentrated H_2SO_4 was added to 0.1 g of dry plant material in the digestion flasks. The reaction solution was left overnight at room temperature. Then 1 mL of 35% H_2O_2 was added to mixture, and digestion flasks were heated in digestion block at 350°C for 30 min. After that, the digestion flasks were removed from the digestion block and 1 mL H_2O_2 was added again. The digestion flasks were again heated at 350°C. The process was repeated until digestion solution appeared colorless. The volume of the mixture was raised to 50 mL with distilled water and filtered. The filtrate was used for the determination of Na^+, K^+, Ca^{2+} and P.

To estimate Na^+, $Ca^{2+}$and K^+, flame photometer (Jenway, model PFP, UK) was used (Allen, 1986). For the P analysis, Jackson (2005) method was followed. The digestion solution (2 mL) was added to 2 mL Barton's reagent, and volume raised to 50 mL with distilled water. The samples were incubated at room temperature, and absorbance read at 470 nm.

Statistical Analysis

Three-factor factorial experiment was performed in completely randomized design (CRD) with four replications for each treatment. Data so obtained were subjected to statistical analyses using Costat 6.2, Cohort software, 2003, Monterey, CA, USA. The difference among means was calculated with the help of Student-Neuman-Keuls test at 5% probability.

Results

Growth Attributes

Salinity significantly decreased shoot and root fresh and dry masses and lengths in both sunflower cultivars. The salt-induced decrease in growth attributes was more in cv. FH-697 than that in cv. FH-687. Biochar treatments (0, 1, 2 and 3% soil amendment) significantly improved all the growth attributes except for root length, which showed non-significant increase because of biochar application. Biochar resulted in the dose-dependent increase in growth attributes as maximal improvement in different growth attributes was recorded in plants supplied with 3% biochar (Fig. 1; Table 2).

Photosynthetic Pigments

Photosynthetic pigments (Chl. *a*, *b*, total Chl. and carotenoids) of sunflower plants were greatly affected upon exposure to salinity. Chl. *a* contents were slightly higher in cv.FH-687 than that of FH-697. Biochar treatments circumvented salinity effects on Chl. *a* and we examined a concentration-dependent increase in Chl. a contents because of biochar application under saline or non-saline conditions. Salinity greatly decreased Chl. *b* contents in both sunflower cultivars. There was no statistical difference between two cultivars for Chl. *b* contents. Biochar application (0, 1, 2, and 3% soil amendment) markedly enhanced Chl. *b* contents under salinity. Soil amendment with 3% biochar yields higher Chl. *b* contents compared with other biochar treatments. Likewise, total Chl. contents did not vary markedly between two cultivars because of biochar application under salinity. Significant decrease in total Chl. contents was recorded in plants subjected to salinity. Biochar application greatly enhanced total Chl. contents and maximal total Chl. values were evident in plants given with 3% biochar application. Carotenoids content decreased substantially in plants upon exposure to salinity. Biochar application caused non-significant increase in carotenoids content in plants under salinity. There was no statistically significant difference between two sunflower cultivars for carotenoids content under saline conditions (Fig. 1; Table 2).

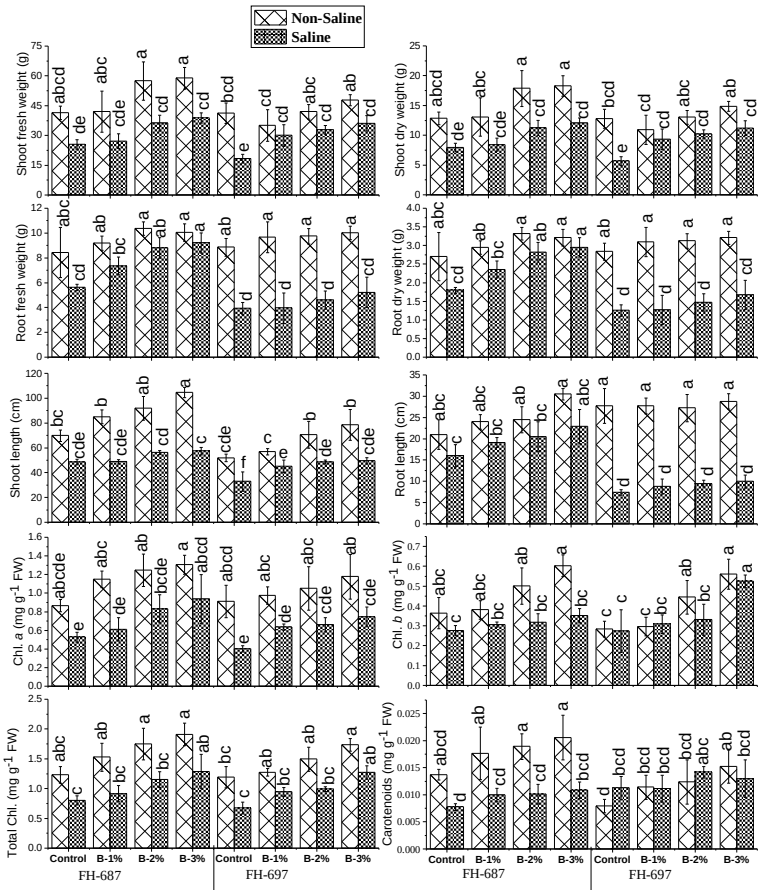

Fig. 1: Growth characteristics and photosynthetic pigments in two sunflower cultivars grown in saline soil amended with varying biochar levels. (*n*= 4; ± S.E.). Abbreviations; B-1%, biochar 1%; B-2%, biochar 2%; B-3%, biochar 3%. Means with different letters are statistically significant at $P \leq 0.05$" please add this statement at the end of all figures legends.

Osmolytes Analysis

Total soluble sugars (TSS), reducing sugars (RS) and non-reducing sugars (NRS): Increase in TSS was more in cv. FH-697 due to salinity, whereas an increase in this variable was not significant in cv. FH-687. The maximal increase in TSS was evident in plants grown in soil with 3% biochar amendment. Salinity significantly decreased RS in both sunflower cultivars. However, decrease in RS due to salinity was more rapid in cv. FH-687 than that in cv. FH-697. Biochar application induced a concentration-dependent increase in RS and this effect of biochar was more visible in cv. FH-697 under salinity. We recorded increase in NRS in sunflower plants. The response of two sunflower cultivars was the same for this variable. Biochar application resulted in further increase in NRS, and maximal values for NRS were observed in plants with 3% biochar under salinity (Fig. 2; Table 2).

TFAA: Salinity decreased TFAA in cv. FH-697, whereas we observed a significant increase in TFAA in cv. FH-687 under salinity. Biochar application enhanced TFAA in both sunflower cultivars, and we found higher increase in this variable in 3% biochar treatment (Fig. 2–3; Table 2).

Organic Osmolytes: To assess the expression of osmotic adjustment in plants under salinity, we measured the endogenous levels of organic osmolytes, namely proline and GB. A sharp increase in the levels of proline and GB was recorded in plants under salinity. Cultivar FH-687 displayed significant higher accumulation of both organic osmolytes as compared to cv. FH-697. Biochar treatments also greatly affected osmolyte accumulation. Plants grown under 3% biochar condition had exhibited maximal osmolyte levels (Fig. 2; Table 2).

Total Soluble Proteins (TSP)

Total soluble proteins were higher in cv. FH-687 than that in cv. FH-697. Salinity significantly increased TSP in sunflower plants. Biochar application induced more increase in TSP. Plants grown in soil with 3% biochar had maximal values for TSP.

Table 2: Mean sum of squares from ANOVA of data for growth and physiochemical attributes of sunflower cultivars grown in soil amended with different biochar levels

S.O.V	Cultivars (CVs)	Salinity (S)	Biochar (B)	CVs×S	CVs×B	S × B	CVs×S×B	Error
	df (1)	*df* (1)	*df* (3)	*df* (1)	*df* (3)	*df* (3)	*df* (3)	*df* (32)
SFW	362.3*	2722.2***	528.2**	100.3^{ns}	32.43^{ns}	44.56^{ns}	57.15^{ns}	84.59
SDW	35.14*	264.1***	51.22**	9.732^{ns}	3.145^{ns}	4.322^{ns}	5.543^{ns}	8.204
RFW	31.74**	143.4***	9.117*	34.46***	1.785^{ns}	0.7005^{ns}	0.5606^{ns}	2.556
RDW	3.251**	14.69***	0.934*	3.531***	0.1828^{ns}	0.0717^{ns}	0.0574^{ns}	0.2618
SL	3096***	9226.8***	1072.1***	634.4*	4.093^{ns}	180.2^{ns}	63.98^{ns}	98.69
RL	183.9*	1771.81***	50.83^{ns}	556.4***	20.71^{ns}	3.058^{ns}	2.352^{ns}	24.61
Chl. a	0.1569^{ns}	2.083***	0.2928^{ns}	0.000014^{ns}	0.0137^{ns}	0.0010^{ns}	0.0179^{ns}	0.104
Chl.b	0.0011^{ns}	0.104*	0.108**	0.038^{ns}	0.008^{ns}	0.011^{ns}	0.0035^{ns}	0.021
Total Chl.	0.184^{ns}	3.117***	0.726**	0.036^{ns}	0.009^{ns}	0.008^{ns}	0.018	0.162
Carotenoids	0.00003^{ns}	0.00016**	0.00005^{ns}	0.00023**	0.000001^{ns}	0.00001^{ns}	0.000002^{ns}	0.00002
RWC	191.77*	27343***	752.5***	201.5*	63.06^{ns}	78.71^{ns}	165.9*	46.02
TSS	10.41^{ns}	126.1^{ns}	4232***	601.4^{ns}	97.11^{ns}	740.2^{ns}	253.5^{ns}	337.8
RS	1.472*	1587***	380.7**	818.2**	13.09^{ns}	11.48^{ns}	69.08^{ns}	63.78
NRS	290.4^{ns}	5312***	1675**	66.63^{ns}	80.01^{ns}	64.77^{ns}	115.46^{ns}	320.3
TFA	68.18***	52.18**	148.52***	123.8***	17.18*	1.976^{ns}	8.571^{ns}	5.111
TSP	48.11**	119.4***	55.42***	45.76**	0.225^{ns}	2.249^{ns}	2.95^{ns}	5.085
Proline	212.6***	1434***	80.59***	0.0016^{ns}	21.29^{ns}	17.23^{ns}	14.11^{ns}	10.27
GB	1867***	3147***	818.8***	0.635^{ns}	21.61^{ns}	90.08^{ns}	67.31^{ns}	50.46
RMP	1062.8***	10955***	533.02***	392.2***	26.59^{ns}	172.1***	38.91^{ns}	17.71
H_2O_2	794.1**	37460***	285.3*	1512.1***	62.62^{ns}	143.7^{ns}	110.37^{ns}	83.87
MDA	370.08**	3982.7***	67.71*	375.5**	9.028^{ns}	48.80^{ns}	6.025^{ns}	30.89
Flavonoids	427.5*	13.84^{ns}	785.7**	7619.8***	11.94^{ns}	2.476^{ns}	133.8^{ns}	144.8
Phenolics	369.4*	1309.1***	623.3***	223.8^{ns}	9.009^{ns}	47.69^{ns}	13.41^{ns}	68.94
Anthocyanins	11.95*	411.5***	57.57***	8.781^{ns}	10.92^{ns}	13.28^{ns}	3.321^{ns}	7.377
AsA	68.01*	1068.6***	389.6***	1252.6***	26.25^{ns}	48.99^{ns}	107.70^{ns}	37.87
SOD	679.4***	4255.3***	493.4***	1.360^{ns}	224.8**	338.2***	287.8^{ns}	44.32
POD	260.39**	4402.7***	59.58^{ns}	195.5**	7.353^{ns}	26.47^{ns}	20.53^{ns}	21.44
CAT	1381**	499.6*	623.4**	1924.3***	247.03^{ns}	56.58^{ns}	58.31^{ns}	114.2
APX	11.35***	13.01***	0.298*	10.20***	0.0547^{ns}	0.435^{ns}	0.109^{ns}	0.306
Root Na	420.1***	21375***	492.6***	320.01***	7.375^{ns}	489.1***	7.322^{ns}	10.54
Stem Na	44.41***	2259.7***	52.08***	33.83***	0.779^{ns}	51.71***	0.774^{ns}	1.114
Leaf Na	905.2***	4787.5***	115.1***	668.09***	31.02***	113.3***	30.96***	4.189
Root K	415.3***	4275***	22.72*	10.741^{ns}	1.376^{ns}	12.61^{ns}	1.488^{ns}	6.763
Stem K	1145.36***	12134***	21.62^{ns}	18.60^{ns}	1.009^{ns}	3.992^{ns}	0.997^{ns}	16.69
Leaf K	200.8***	1583.2***	29.69***	1.188^{ns}	0.0680^{ns}	23.45***	0.00040^{ns}	3.251
Root Ca	729.7***	1063.8***	29.11**	94.62***	2.588^{ns}	11.41^{ns}	1.014^{ns}	6.49
Stem Ca	449.6***	655.5***	17.93**	58.31***	1.595^{ns}	7.028^{ns}	0.625^{ns}	3.999
Leaf Ca	1798.7***	2622.2***	71.74***	233.2***	6.381^{ns}	28.11^{ns}	2.501^{ns}	15.99
Root P	4.496***	6.555***	0.221**	0.583***	0.0196^{ns}	0.0286^{ns}	0.0025^{ns}	0.0399
Stem P	0.0628***	0.0916***	0.00308**	0.0081***	0.00027^{ns}	0.000401^{ns}	0.000035^{ns}	0.00055
Leaf P	1.571***	2.291***	0.077***	0.2037***	0.0068^{ns}	0.010^{ns}	0.00089^{ns}	0.0139

ns, non-significant; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, non-significant; df, degree of freedom; SFW, shoot fresh weight; SDW, shoot dry weight; RFW, root fresh weight; RDW, root dry weight; SL, shoot length; RL, root length; Chl. a, chlorophyll a; Chl.b, chlorophyll b; Total Chl., total chlorophyll; LRWC, leaf relative water content; TSS, total soluble sugars; RS, reducing sugars; NRS, non-reducing sugars; TFA, total free amino acids; TSP; total soluble protein; GB, glycine betaine; RMP, relative membrane permeability; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; AsA, ascorbic acid; SOD, superoxide dismutase; POD, peroxidase; CAT, catalase; APX, ascorbate peroxidase; Na, sodium; K, potassium; Ca, calcium; P, phosphorous

Membrane Permeability, Oxidative Damage and Antioxidants

Oxidative stress indicators: Measurements of relative membrane permeability (RMP) revealed a significant increase because of salinity. Higher RMP was observed in cv. FH-697 than that of cv. FH-687 under tested conditions. A significant decrease in RMP was detected in salinity stressed plants due to biochar application. Plants supplied with 3% biochar showed a more significant decrease in RMP under stress conditions. We quantified the changes in H_2O_2 in plants upon exposure to salinity. A significant increase in H_2O_2 was seen in cv. FH-697, while H_2O_2 levels were lower in cv. FH-687. Biochar treatment displayed decline in H_2O_2 contents and plants from 3% biochar treatment exhibited minimal H_2O_2 values under saline conditions. Similar to the salinity effect on H_2O_2 accumulation, a marked increase in MDA levels was seen in plants due to salinity. NaCl-induced increase in MDA was more considerable in cv. FH-697, while comparatively lower MDA levels were detected in cv. FH-687. Maximal decline in MDA contents was evident in plants when 3% biochar was used as soil amendment (Fig. 3; Table 2).

Non-enzymatic antioxidants: In order to characterize the oxidative defense mechanism, we measured the concentrations of different non-enzymatic antioxidants. Salinity-induced significant increase in flavonoids was detected in cv. FH-697, while a decrease in this variable was

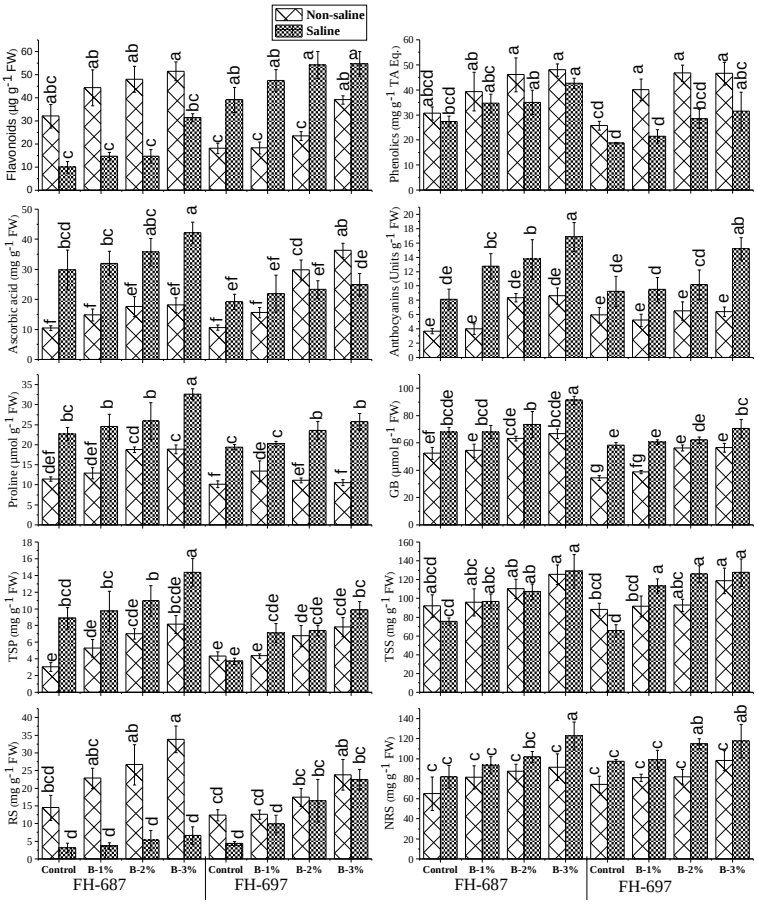

Fig. 2: Changes in key biochemical attributes in two sunflower cultivars grown in saline soil amended with varying biochar levels. (*n*= 4; ± S.E.). Abbreviations; GB, glycine betaine; TSP, total soluble protein; TSS, total soluble sugars; RS, reducing sugars; NRS, non-reducing sugars; B-1%, biochar 1%; B-2%, biochar 2%; B-3%, biochar 3%. "Means with different letters are statistically significant at P ≤ 0.05" please add this statement at the end of all figures legends.

displayed by cv. FH-687. A dose-dependent increase in flavonoids was seen due to biochar treatments. Plants from 3% biochar application had significantly more flavonoids under tested conditions. Our results indicated a significant decrease in phenolics under salinity. Higher phenolics content were seen in cv. FH-687 than that in cv. FH-697. Plants with 3% biochar treatment displayed a more significant increase in phenolics under saline or non-saline conditions. We recorded salt-induced increase in anthocyanins in plants upon exposure to salinity. Cultivar FH-687 showed higher anthocyanins than that of cv. FH-697. Significant increase in anthocyanins was evident in plants due to 3% biochar soil amendment under salinity. Measurements of ascorbic acid (AsA) contents in plants subjected to salinity revealed significant increase in AsA in cv. FH-687, while decrease in AsA contents was detected in cv. FH-697. Biochar application yielded a concentrations-dependent increase in AsA contents. We recorded greater AsA contents in plants with 3% biochar soil amendment (Fig. 2; Table 2).

Antioxidant enzymes activities: In general, to overcome oxidative damage caused by increased cellular levels of H_2O_2 and MDA, plants display variations in antioxidant enzyme activities. Data given in figure 5 clearly showed salt-induced increase in SOD activity. Greater SOD activity was detected in cv. FH-687, while cv. FH-697 was inferior in this context. Biochar treatments displayed increase in SOD activity which was maximal in plants with 3% biochar amendment of soil. POD activity increased markedly in salinity stressed plants. Higher POD activity was seen in cv. FH-687, while cv. FH-697 displayed lower POD activity. Further increase in POD activity was detected in plants from 3% biochar treatment under salinity. CAT activity was variable between the cultivars under salinity. Increase in CAT activity was measured in cv. FH-687, while cv. FH-697 exhibited decline in this variable under salinity. The maximal CAT activity was seen in plants with 3% biochar treatment under stress conditions. Salt-induced increase in APX activity was seen in cv. FH-687 and this increase was even more in plants grown in soil with 3% biochar application. Conversely, cv. FH-697 did not have significant changes in APX activity except for 3% biochar application,

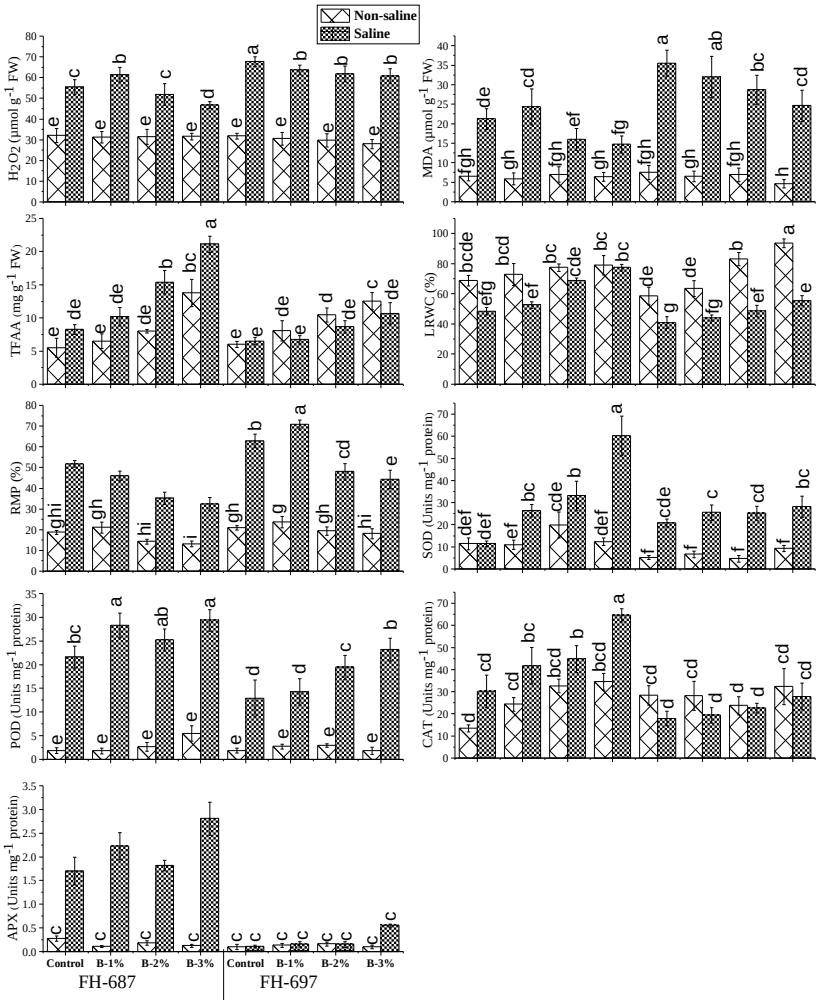

Fig. 3: Changes in key biochemical attributes in two sunflower cultivars grown in saline soil amended with varying biochar levels. (*n*= 4; ± S.E.). Abbreviations; TFAA, total free amino acids, LRWC, leaf relative water contents; RMP, relative membrane permeability; B-1%, biochar 1%; B-2%, biochar 2%; B-3%, biochar 3%. "Means with different letters are statistically significant at $P \leq 0.05$" please add this statement at the end of all figures legends.

where we recorded conspicuous increase in APX activity (Fig. 3; Table 2).

Ionic and Nutrient Contents

Plant Na^+ contents: Salinity significantly increased root, stem and leaf Na^+ content in both sunflower cultivars. Higher Na^+ content in different plant parts was detected in cv. FH-697 than that in cv. FH-687. There was gradual decline in plant Na^+ content with increase in soil biochar level. Plants with 3% biochar had minimal values for tissue Na^+ (Fig. 4; Table 2).

Plant Ca^{2+} contents: Exposure of plants to salinity significantly increased root, stem and leaf Ca^{2+} contents. The response of two sunflower cultivars was not the same for Ca^{2+} in different plant parts. Higher plant Ca^{2+} levels were displayed by cv. FH-687, while cv. FH-697 was inferior with respect to plant Ca^{2+} contents. A concentration-dependent increase was seen in plant Ca^{2+} because of biochar application (Fig. 4; Table 2).

Plant K^+ contents: Salinity induced a significant decrease in root, stem and leaf K^+ contents. Significant difference was observed for root, stem and leaf K^+ between two sunflower cultivars. Higher K^+ contents in different plant parts were observed in cv. FH-687 than that in cv. FH-697. Biochar application markedly enhanced plant tissue K^+ and this effect of biochar was evident in plants with 3% biochar application (Fig. 4; Table 2).

Plant P contents: The amount of P in root, stem and leaf was several folds higher in biochar treated plants than in control plants. Cultivar FH-687 showed considerably greater plant P contents than that of cv. FH-697. Higher doses of biochar in soil resulted in higher P levels in different plant parts (Fig. 4–5; Table 2).

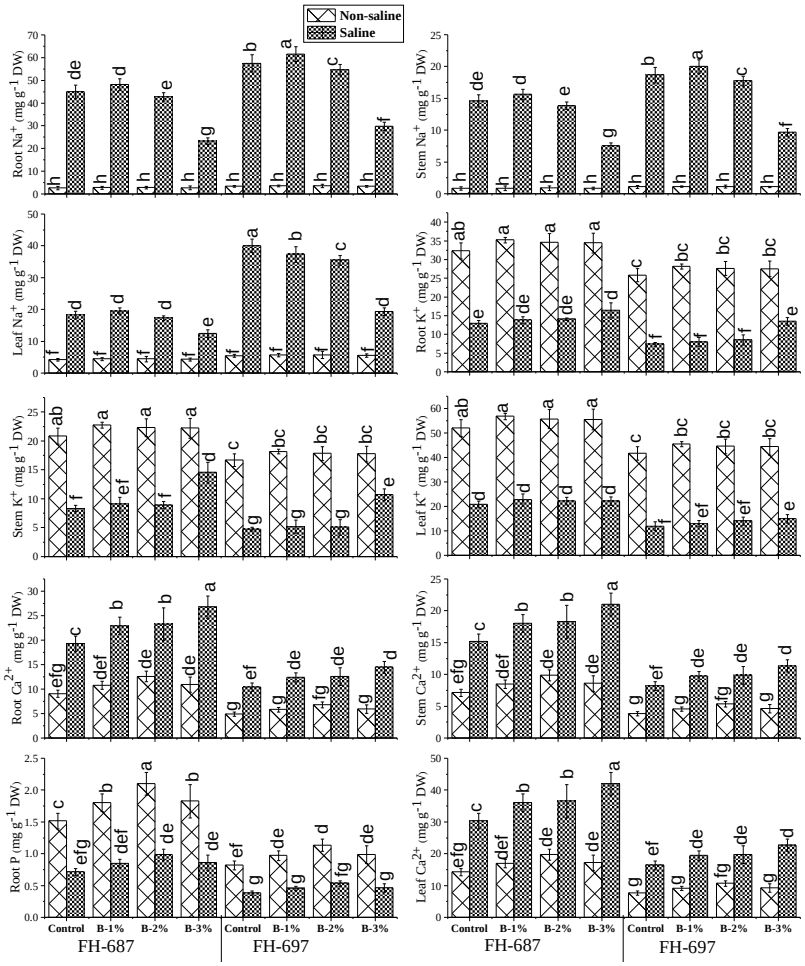

Fig. 4: Changes in Na^+, K^+, Ca^{2+} and P contents in different plant parts in two sunflower cultivars grown in saline soil amended with varying biochar levels. (*n*= 4; ± S.E.). Abbreviations; B-1%, biochar 1%; B-2%, biochar 2%; B-3%, biochar 3%. "Means with different letters are statistically significant at $P \leq 0.05$" please add this statement at the end of all figures legends.

Discussion

In present study, salinity decreased growth, RWC and chlorophyll in sunflower cultivars due to higher tissue concentration of toxic Na and lower levels of tissue K (Fig. 4; Table 2). Higher levels of Na in cells resulted in enhanced generation of toxic ROS (e.g., H_2O_2) and lipid peroxidation product (MDA). Growth reduction in sunflower was ascribed to chlorophyll degradation due to oxidative and ionic stresses of salinity (Fig. 1, 4; Table 2). We recorded a significant decline in photosynthetic pigments in salinity stressed sunflower plants. The decline in photosynthetic pigments was attributed to their enhanced degradation and limited biosynthesis under salinity (Farhangi-Abriz and Torabian, 2018). Biochar application may increase plant growth and photosynthetic pigments by improving mineral nutrients availability (K, P, Mg, Ca and S), whereas higher photosynthetic pigments were due to biochar-mediated improvement in biological and physicochemical characteristics of soil (Farhangi-Abriz and Torabian, 2018). Solaiman *et al.* (2012) reported that an increase in growth of beans by soil biochar application was due to the influence of biochar on soil pH and nutrient availability. In our study, biochar application (3%) significantly enhanced K and decreased Na contents that could have been the main contributing factor for higher plant growth and lower degradation of photosynthetic pigments. Furthermore, the results of the present study revealed lower lipid peroxidation, H_2O_2 production, and RMP in plants with (3%) biochar application. Plants with more ROS generation under salinity exhibited more damage to photosynthetic pigments and membranes as evident in the form of more MDA and RMP levels in this study (Kaya *et al.*, 2018). Cultivar FH-687 had lesser salt-induced decrease in growth and photosynthetic pigments than that in cv. FH-697. Cultivar FH-687 displayed minimal oxidative damage and specific ion toxicity that might have resulted in better salinity tolerance compared with cv. FH-697.

There exists a dynamic equilibrium between the production and detoxification of ROS such as H_2O_2, $O_2^{\cdot-}$,

and $OH^{\cdot}$ produced via several metabolic pathways. This equilibrium shook because of salinity that triggers the generation and accumulation of ROS (Rasheed *et al.*, 2018). Excessive tissue ROS levels may cause oxidative stress that damages proteins, DNA, membranes, and pigments (Ashraf *et al.*, 2018). Enhanced lipid peroxidation due to higher cellular concentration of ROS cause cell dysfunction and death (Rasheed *et al.*, 2018). Our study also revealed salinity mediated more lipid peroxidation and loss of membrane integrity in the form of MDA and RMP, respectively. However, a continuous check on tissue ROS levels is present in the form of enhanced activities of antioxidant enzymes such as CAT, APX, POD, and SOD (Rasheed *et al.*, 2018). In our study, biochar application decreased ROS generation and accumulation that subsequently limited lipid peroxidation with the help of enhanced activities of antioxidant enzymes. We also recorded salt-induced decrease in the accumulation of low molecular weight non-enzymatic antioxidants such as phenolics, flavonoids, and increase in ascorbic acid and anthocyanins. Cultivar FH-687 was superior to cv. FH-697 for cellular levels of non-enzymatic antioxidant compounds. Non-enzymatic antioxidant compounds play significant part in safeguarding plants from ROS-induced oxidative stress (Hasanuzzaman *et al.*, 2018). Our results revealed increase in the endogenous levels of ascorbic acid and anthocyanins in plants with biochar application. Ascorbic acid and anthocyanin possess potent antioxidant activity that enables them to play pivotal part in plant abiotic stress tolerance. Plants tend to accumulate enhanced levels of anthocycanins and ascorbic acids under abiotic stresses (Khoo *et al.*, 2017).

Results of the present study displayed increase in soluble sugars, proteins, amino acids, reducing sugars, proline and glycine betaine in salinity stressed plants. Osmotic adjustment is a crucial phenomenon in plants that enable plants to tolerate salinity in the growth medium (Ashraf *et al.*, 2018). Plants accumulate solutes in large amount to lower cell water potential to maintain cell turgor under salinity as evident in the present study where plants accumulated more GB, proline, sugars, soluble proteins and amino acids (Ashraf *et al.*, 2015). GB is an efficient osmoprotectant accumulated in diversity of plant species in response to stress. Likewise, increase in proline, and soluble amino acids are related to osmotic adjustment (Farhangi-Abriz and Torabian, 2018). Free amino acids also act as osmoprotectants and decrease osmotic potential of cells under stress conditions (Ashraf *et al.*, 2015). In addition to proline functions as osmoprotectant, it also acts as O_2^{-}, and $OH^{\cdot}$ scavenger, inhibitor of lipid peroxidation, metal chelator, and protein stabilizer. Accumulation of osmolytes performs two possible physiological roles: (i) stabilize membranes and (ii) decrease osmotic potential of cell in order to maintain cell turgor (Buchanan *et al.*, 2000).

Excessive tissue Na concentration causes specific ion toxicity, which inhibits osmotic regulation, and disrupts nutrient balance (Katerji *et al.*, 2004). Reduction in tissue K concentration was result of exchange of K with Na ions under salinity (Fig. 4). The decrease in tissue K content induced by Na is a well-known competitive process exhibited by plants under salinity (Ashraf *et al.*, 2015; Ashraf *et al.*, 2018). Salinity increases external Na concentration that not only interferes with the uptake of other nutrients but also damages root membranes altering their selectivity (Ashraf *et al.*, 2018).

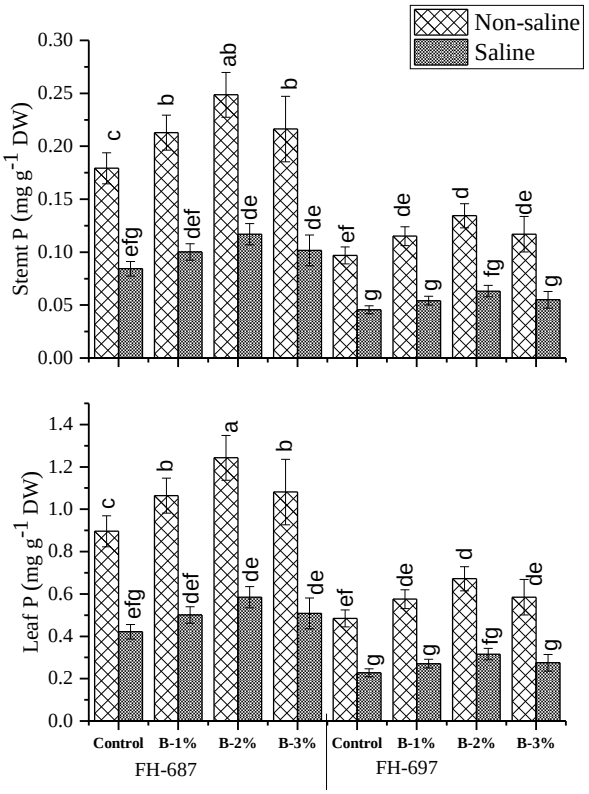

Fig. 5: Changes in P contents in different plant parts in two sunflower cultivars grown in saline soil amended with varying biochar levels. (n= 4; ± S.E.). Abbreviations; B-1%, biochar 1%; B-2%, biochar 2%; B-3%, biochar 3%. Means with different letters are statistically significant at $P \leq 0.05$" please add this statement at the end of all figures legends.

Salinity alters soil cation exchange capacity, which might affect the uptake of other nutrients such as Ca, K, Mg, Zn and Fe (Ashraf *et al.*, 2018). In addition, biochar affects buffering and ion exchange capacity, nutrient cycling, and availability and soil pH that in turn increase the uptake of nutrients such as K (Farhangi-Abriz and Torabian, 2018). Laird *et al.* (2010) also reported an increase in K contents of plants grown in biochar added soil. The increase in nutrients may have been due to the greater immobilization of nutrients via adsorption reaction after soil biochar application (Beesley *et al.*, 2011). In our study, higher P contents may be attributed to the enhanced availability of P in saline soils (Turan *et al.*, 2010). Biochar indicates the potential to reclame saline land. Biochar soils exhibit more water holding capacity than non-biochar soils, which could

be an added advantage for plants growing under salinity. In our study, we also recorded more RWC in plants with added biochar in soil. Our results are in parallel with reported response of salinity stressed plants for RWC after soil biochar application (Farhangi-Abriz and Torabian, 2018).

We recorded a significant increase in Na in different plant parts under salinity. However, incorporation of biochar in soil markedly decreased plant Na contents. Biochar, being more stable in soil, decreases Na uptake and thereby significantly contribute towards mitigation of salinity (Iqbal *et al.*, 2018). Akhtar *et al.* (2015) reported alleviation of salinity in potatoes by soil biochar application since biochar had enormous adsorption potential for Na. Likewise, Thomas *et al.* (2013) also reported high Na adsorption in soil with biochar incorporation. Similarly, Farhangi-Abriz and Torabian (2018) also reported biochar mediated alleviation of salinity in beans in the form of minimal tissue Na buildup.

Conclusion

Soil biochar amendment ameliorated salinity stress in sunflower through Na^+ adsorption that in turn enhanced the availability of other nutrients and improved growth and physiochemical attributes. Biochar-induced salinity tolerance was associated to oxidative defense system, osmotic adjustment, and reduced specific ion toxicity. Cultivar FH-687 showed higher growth due to lower oxidative damage and specific ion toxicity. Biochar effects may vary with the source of biochar and plant species. Therefore, further field trials are needed to fully understand the potential of biochar to circumvent salinity effects on plant growth in salt-affected soils.

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