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AMINOLEVULINIC ACID-INDUCED CHANGES IN YIELD AND SEED-OIL CHARACTERISTICS OF SUNFLOWER (*HELIANTHUS ANNUUS* L.) PLANTS UNDER SALT STRESS

NUDRAT AISHA AKRAM[1*], MUHAMMAD ASHRAF[2,3], AND F. AL-QURAINY[3]

[1]*Department of Botany, GC University, Faisalabad, Pakistan*
[2]*Department of Botany, University of Agriculture, Faisalabad, Pakistan*
[3]*Department of Botany & Microbiology, King Saud University Riyadh, Saudi Arabia*
*Corresponding author's email: nudrataauaf@yahoo.com

Abstract

Effectiveness of a potential plant growth regulator, 5-aminolevulinic acid (ALA) on improving yield and regulating some potential physio-chemical attributes of seed and oil of two sunflower cultivars was examined in a greenhouse experiment. Four varying levels of ALA 20, 50 and 80 mg L^{-1} were applied foliarly to the two sunflower cultivars (Hysun-33 and S-278) grown under saline (150 mM NaCl) and non-saline (0 mM NaCl) conditions. Salt stress adversely affected the achenes/plant, total achene yield, 100-achene weight, seed moisture content, seed oil percentage, seed K^+ and P concentrations of both sunflower cultivars, while in contrast, salt-induced increase in the accumulation of Cl^- and Na^+ in seed as well as α-tocopherols in oil of both sunflower cultivars. However, no significant change in seed organic and inorganic contents, oil refractive index (RI) and seed Ca^{2+} content was observed due to salt stress in both sunflower cultivars. Foliar-applied varying levels ALA remained ineffective in improving all yield variables, oil refractive index and seed inorganic nutrients (Na^+, Cl^-, K^+ and P) of both sunflower cultivars under saline and non-saline regimes. However, a significant increase in seed oil concentration, accumulation of oil α-tocopherols and seed P of both sunflower cultivars was observed due to foliar-applied 20 and 80 mg L^{-1} of ALA under saline conditions. Overall, ALA was ineffective in ameliorating the salt-induced adverse effects on yield and different seed-oil characteristics except of seed P and oil α-tocopherols in sunflower plants.

Introduction

Crop yield is one of the important criteria for appraising salinity tolerance in plants as it is the final output of all the interrelated growth and vital physiological attributes negatively influenced by the imposition of saline stress (Katerji *et al*., 2003; Flowers, 2004; Munns *et al.,* 2006; Flowers *et al.,* 2010; Ashraf *et al.,* 2010a, b). Reduction in crop growth takes place due to the interplay of a myriad of processes taking place at molecular, cellular and whole plant levels (Ashraf & Akram, 2009; Noreen & Ashraf, 2010). When a plant is exposed to saline stress all the vital processes including photosynthesis, protein synthesis, nutrient uptake, membrane permeability, osmotic and energy balance, lipid metabolism and oxidative defensive system are severely perturbed (Ashraf, 2009; Flowers *et al*., 2010). For example, more recently, reported that salinity stress significantly decreased growth and yield of *Solanum melongena* due to disturbance in photosynthesis, sub-stomatal CO_2 and stomatal regulation (Abbas *et al*., 2010). Similarly, suppression in yield as well as growth production of sunflower plants was observed due to reduction in chlorophyll pigments, quantum yield (F_v/F_m), and K^+/Na^+ ratio (Akram & Ashraf, 2011). In another study, salt stress markedly reduced achene yield and oil content of two sunflower cultivars (Noreen & Ashraf, 2010), while in contrast, Ahmad *et al*. (2007) did not find salinity-induced changes in seed yield and composition of seed oil in cotton, however, tocopherols and fatty acid profiles of the cotton seed oil were significantly affected.

It is widely reported that genetic and environmental factors can affect the oil yield and quality of its seed/grain production (Ali & Ashraf, 2011). Saline soils can markedly reduce seed oil content of sunflower achenes (Szabolcs, 1994). In addition, salt-induced alteration in tissue/organ nutrient contents is one of the common phenomena taking place in most plants grown under saline regimes (Munns *et al*., 2006; Akram & Ashraf, 2011a). Although considerable salt-induced changes in nutrient contents in plant roots and shoots have been widely reported, studies on salt-induced changes in seeds are relatively less reported in the literature.

The potential plant growth regulator, 5-aminolevulinic acid (ALA), is an important biosynthetic precursor of all tetrapyrroles such as vitamin B_{12}, billins, heme, chlorophyll and other specialized machinery in plants as well as animals (Rebeiz *et al*., 1984; von Wettstein *et al*., 1995; Akram & Ashraf, 2011a, b). Exogenous application of ALA is considered as an effective means of minimizing the salt-induced adverse effects in a number of crops e.g., pakchoi (*Brassica campestris*) (Wang *et al*., 2005), date palm (*Phoenix dactylifera*) (Youssef & Awad, 2008), oilseed rape (*Brassica rapa*) (Naeem *et al*., 2010), spinach (*Spinacia oleracea*) (Nishihara *et al*., 2003) and potato (*Solanum tuberosum*) (Zhang *et al*., 2006). ALA is known to regulate several key physiological processes associated with plant growth under saline regimes (Hotta *et al*., 1997a, b) such as seed germination, reduced Na^+ uptake, altered light reactions, improved reactive oxygen species scavenging, enhanced photosynthetic assimilation and maintenance of nutrient status (Hotta *et al*., 1997a, b; Youssef & Awad, 2008). Similarly, ALA application increased the yield of garlic, barley, rice and potato plants by significantly enhancing the photosynthetic capacity and plant biomass (Tanaka *et al*., 1992). ALA has been also reported to be effective in improving plant growth, and crop yields as well as carbon and nitrogen fixing processes (Maruyama-Nakashita *et al.,* 2010).

Keeping in view all the earlier mentioned reports it was hypothesized that exogenous application of ALA not only alters growth and physiological processes, but it also

can change the seed composition of salt-stressed sunflower plants. Thus, the major aim of the present study was to examine whether foliar-applied different levels of ALA could improve seed yield and alter seed composition as well as seed oil quality in plants of two sunflower cultivars under saline stress.

Materials and Methods

A greenhouse experiment was conducted during February-May, 2009, to assess the effect 5-aminolevulinic acid (ALA) on yield and yield components and some physio-chemical attributes of seed and oil contents of sunflower plants grown under non-saline and saline conditions. The detail of the experiments has already been presented by Akram & Ashraf (2011a). Plants were irrigated for two weeks with full strength Hoagland's nutrient solution. After three weeks of plant growth, two salt treatments, no salt (control) and 150 mM NaCl were applied to the rooting medium. Each pot was supplied weekly with 2 L of the salt treatment solution. During the week, the sand in each pot was daily moistened with 250 mL distilled water. Four levels of ALA (MP Biomedical Inc., Mol. wt = 167.59), i.e., control (non-spray), 20, 50 and 80 mg L^{-1} prepared in 0.1% tween-20 were applied as a foliar spray (35 mL per pot) to control (non-stressed) and NaCl-stressed sunflower plants of both lines. At maturity, data for number of achenes per plant, achene yield/plant and hundred-achene weight were recorded by removing the heads from mature sunflower plants. Data for the following attributes were determined:

Moisture contents: After recording the fresh weights of the achenes, they were placed in an oven at 60°C for 72 h to dry them after which time dry weights were recorded. The moisture contents were calculated as:

Achene moisture contents (%) = Achene fresh weight – Achene dry weight x 100

Organic and inorganic nutrients (%): The oven-dried achenes were milled into fine powder using an electric grinder. To obtain ash (inorganic contents), finely ground 1 g material was taken in a crucible dish and then transferred into a muffle furnace at 550°C for 4 h. After cooling the ash was weighed which was equal to the inorganic contents. Organic contents were determined as:

Organic contents = 1 - inorganic contents + moisture contents

Inorganic nutrients: The oven-dried chopped seed material (100 mg) was digested in 10 mL Pyrex glass vials with 2 mL digestion mixture [14.0 g $LiSO_4$ (BDH Chemicals Ltd. Poole , England) + 0.42 g Se + 350 mL H_2O_2 (35% A.R. Grade extra pure)] and 0.5 mL perchloric acid ($HClO_4$), kept on a hot plate enclosed in a fume hood. Temperature of the hot plate was maintained at 350°C. The digestion of the material was considered complete when the material became colorless. Final volume of the extract was raised to 50 mL with re-distilled H_2O, filtered and used for the determination of sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), and phosphorus (P) concentrations following Allen *et al.* (1986).

Determination of Ca^{2+}, K^+ and Na^+: Using a single channel flame photometer (Jenway, PFP-7, UK) Ca^{2+}, K^+ and Na^+ were analyzed with extra-pure A grade series of standards (5 to 60 mg L^{-1}). The standard curves were drawn for each element and the concentrations of Ca^{2+}, K^+ and Na^+ worked-out using appropriate standard curves.

Phosphorus (P): Barton's reagent was prepared following Jackson (1962) by dissolving 12.5 g of ammonium molybdate in 200 mL of H_2O in a beaker. Then in another beaker 0.625 g ammonium metavandate was dissolved in 300 mL hot water, cooled and 250 mL of conc. HNO_3 added to it. Both solutions were cooled and mixed carefully and the final volume maintained up to 500 mL. The filtrate (1 mL) was mixed in 1 mL Barton's reagent and total volume brought to 25 mL with distilled H_2O. These samples were kept for 30 min prior to reading on a spectrophotometer at 470 nm. The values of P were calculated using a standard curve.

Determination of Cl^-: Chloride determination in dried seed was carried-out through water extraction. The oven-dried material for each sample (0.1 g) was taken in a 25 mL test tube. Then, 10 mL distilled water were added to it and the tubes then heated at 80°C on a hot plate for 2 h. The final volume of each sample was maintained up to 10 mL by adding de-ionized distilled water. A chloride analyzer (Model 926, Sherwood Scientific Ltd., Cambridge, UK) was used to determine Cl^- concentration in the extracts.

Extraction of oil: Following the IUPAC standard method (1979), the ground seed material was used for oil extraction with the help of 500 mL *n*- hexane (Mol. wt. 86.18; Merck, Darmatadt, Germany, HPLC grade) in a Soxhlet apparatus. The ground seeds were filled in a thimble and the open side of the thimble covered with wool. The thimble was fed into the Soxhlet extractor. The oil extraction was completed after 10 h. The *n*-hexane was aspirated using a vacuum pump at 45°C. The oil was separated into glass bottles and stored in a refrigerator for different analyses.

Refractive index (RI): To appraise RI, the procedure of AOAC (1984) was adopted using the Abbe's refractometer (Model 922313; Bellingham and Stanley Ltd. London). Refractive index was read after putting a drop of oil on the prism. During this, the temperature of the prism was adjusted at 40°C.

$$n = \frac{\text{Sin } i}{\text{Sin } r} = \frac{\text{Velocity of light in air}}{\text{Velocity of light in liquid}}$$

Oil contents: The oil contents were calculated as:

Oil contents (%) = Oil contents (g)/seed weight (g) x 100

Determination of α-tocopherols: A high performance liquid chromatography (HPLC) [Sykram GmbH, Kleinsthein, Germany] fitted with a UV detector (Model: S- 3210) was used for the determination of alpha-tocopherols following Lee *et al*. (2003). One g oil was mixed in 0.05 g ascorbic acid, 5 mL ethanol (90.2%) and

0.5 mL KOH (80%) in a 10 mL flask and vortexed it for 30 min. Then, each flask was placed in a water bath at 70°C for half an hour, thereafter they were cooled at room temperature. To each flask containing oil sample, 3 mL deionized water and 5 mL *n*-hexane were added and they were vortexed again. All aliquots were centrifuged at 1000 x *g* for 10 min at normal temp and transferred the upper *n*-hexane layer into another flask and re-extracted with the similar method. The material was filtered and an aliquot of 20 µL was injected into a Hypersil ODS reverse phase (C-18) guard column. The solvents containing HPLC-grade acetonitrile and methanol (35:65 v/v) were used for elution system. The elution mixture at a flow rate of 1.3 mL/min was used to separate the chromatic portion. The OD of separated solution was read at 292 nm. The identification of α-tocopherols was done by comparing the retention time. Data were calculated using the computer SRI peak simple chromatography (SRI Instrument Torrance, California USA).

Experimental design and statistical analysis: The experiment was framed-up in a three-factor factorial completely randomized design (CRD). Bartlett's test for analysis of variance (ANOVA) of the data was performed for each dependant or independent variable computed using the MSTAT computer package (MSTAT Development Team, 1989).

Results

Yield and yield-related attributes of both sunflower cultivars (Table 1; Fig. 1) showed that imposition of 150 mM of salt stress adversely affected achenes/plant, total achene yield and 100-achene weight of both cultivars. In contrast, foliar-applied varying levels of ALA remained ineffective in improving all the yield variables of both cultivars of sunflower under study. Similarly, no significant difference was observed between both cultivars with respect to yield variables.

A considerable reduction in seed moisture contents was observed under saline conditions. In contrast, salt stress did not affect the seed organic as well as seed inorganic contents (Table 1; Fig. 1). Both sunflower cultivars were almost similar in seed moisture contents, seed organic along with seed inorganic contents under stress as well as non-stress conditions.

Table 1. Mean squares derived from analysis of variance of data for yield and some physio-chemical characteristics of seed and oil of salt-stressed and non-stressed sunflower (*Helianthus annuus* L.) plants subjected to foliar-applied varying levels of 5-aminolevulinic acid (ALA).

Source of variation	df	Achenes/plant	Achene yield/plant	100-achene weight	Seed moisture content	Seed organic contents
Salinity (S)	1	572854.1***	358.4***	0.108***	9.881***	0.519ns
Cultivars (Cvs)	1	37.65ns	0.843ns	0.001ns	0.348ns	25.01ns
ALA	3	217.8ns	0.514ns	0.0025ns	1.776ns	38.38ns
S x Cvs	1	21360.9*	3.85ns	0.002ns	0.101ns	2.35ns
S x ALA	3	5910.3ns	0.87ns	0.0174*	0.051ns	64.76ns
Cvs x ALA	3	8930.4ns	2.17ns	0.003ns	1.914*	40.07ns
S x Cvs x ALA	3	3494.4ns	2.03ns	0.004ns	0.375ns	15.053ns
Error	48	3830.5	1.131	0.006	0.64	30.048
	df	**Seed inorganic contents**	**Oil contents**	**Oil refractive index**	**Oil α-tocopherols**	**Seed Na^+**
Salinity (S)	1	2.847ns	1439.2***	0.0004ns	645.6***	597.2***
Cultivars (Cvs)	1	0.003ns	8.724ns	0.0012ns	16.13*	41.44ns
ALA	3	5.587ns	237.2***	0.0003ns	14.96**	24.54ns
S x Cvs	1	10.16ns	159.9*	0.0003ns	0.108ns	145.5*
S x ALA	3	1.701ns	14.16ns	0.0005ns	62.67***	26.58ns
Cvs x ALA	3	4.358ns	120.95*	0.0003ns	29.82***	18.587ns
S x Cvs x ALA	3	1.681ns	60.04ns	0.00343ns	6.946*	34.77ns
Error	48	7.154	29.63	0.0026	2.354	22.16
	df	**Seed Cl^-**	**Seed K^+**	**Seed Ca^{2+}**	**Seed P**	
Salinity (S)	1	2104.5***	870.25***	31.64ns	0.0027**	
Cultivars (Cvs)	1	2.64ns	33.062ns	21.39ns	0.002*	
ALA	3	32.47ns	24.42ns	0.432ns	0.001*	
S x Cvs	1	4.515ns	1.562ns	2.64ns	0.004***	
S x ALA	3	33.68ns	20.5ns	12.39ns	0.003***	
Cvs x ALA	3	125.2*	28.73ns	3.55ns	0.002**	
S x Cvs x ALA	3	41.18ns	54.23ns	0.223ns	0.0007ns	
Error	48	31.25	28.26	8.619ns	0.0002	

ns = non-significant; *, ** and *** = significant at 0.05, 0.01 and 0.001 levels, respectively

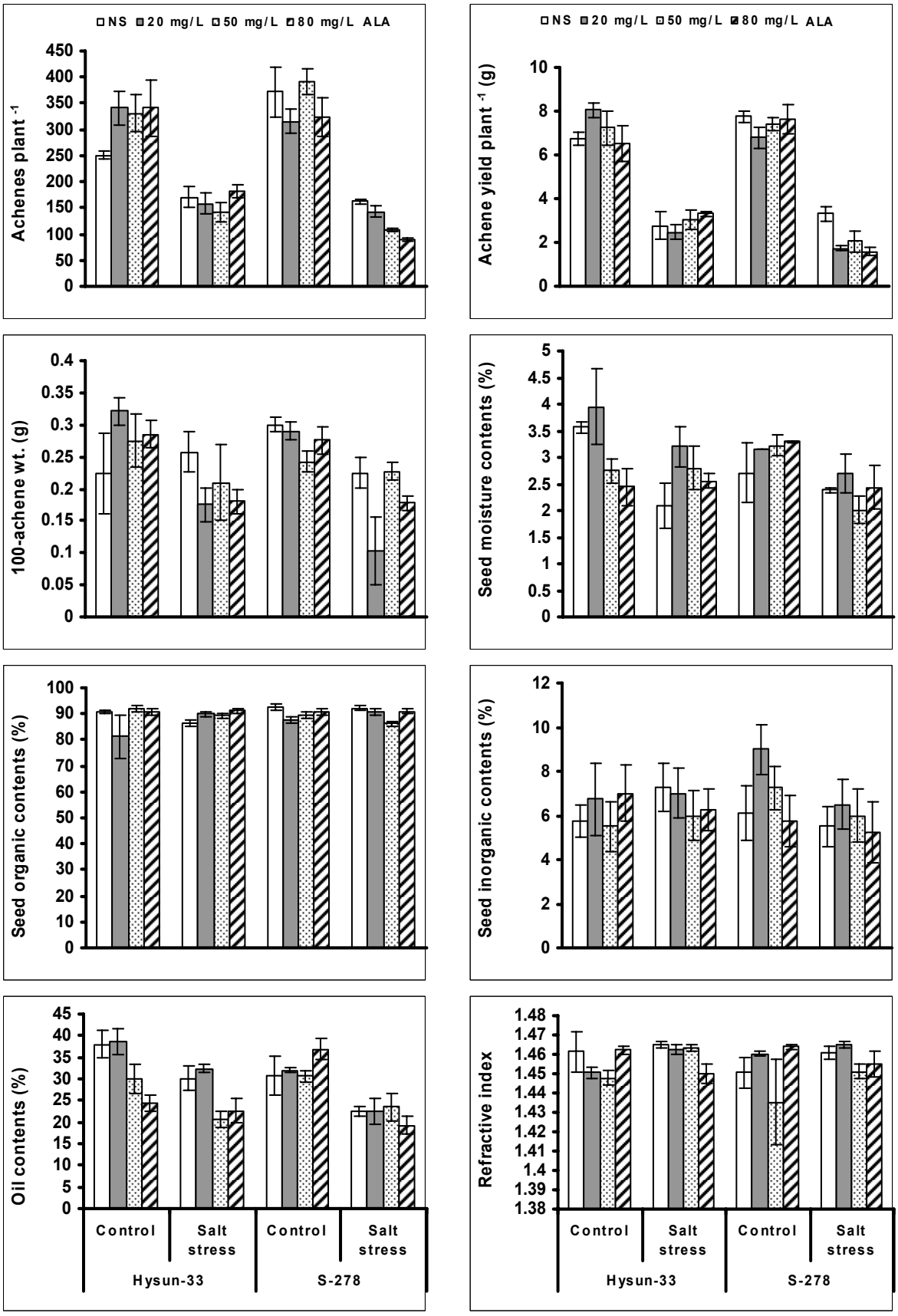

Fig. 1. Yield and yield components, physical properties of seed oil and percentage of oil of salt-stressed and non-stressed plants of two cultivars of sunflower (*Helianthus annuus* L.) subjected to foliar-applied varying levels of 5-aminolevulinic acid (Mean ± S.E.). NS stands for No Spray.

Seed oil percentage (Table 1; Fig. 1) of both cultivars decreased due to NaCl application, although almost a similar trend of reduction in oil contents (%) was observed in both sunflower cultivars. A little or no increase in seed oil concentration was observed due to ALA under saline conditions but in contrast a considerable decrease observed in seed oil concentration under saline conditions in response to application of 50 and 80 mg L^{-1} ALA to both sunflower cultivars (Fig. 1).

Refractive index (RI) (measured by using a refractometer) of oil of both sunflower cultivars grown under non-saline and saline regimes remained unaffected due to salt stress (Fig. 1). Both cultivars were similar in RI and foliar-applied varying levels of ALA did not alter the reflectance of sunflower oil.

Under salt stress, a marked increase in α-tocopherols in oil of both sunflower cultivars was explored (Table 1; Fig. 2). Of both sunflower cultivars, cv. Hysun-33 was relatively ($p \leq 0.05$) higher in α-tocopherols than the cv. S-278 particularly under saline conditions. Foliar-applied different levels of ALA significantly ($p \leq 0.001$) enhanced the accumulation of α-tocopherols in both sunflower cultivars under saline regimes and most effective level of ALA was found to be 20 mg L^{-1} under NaCl stress.

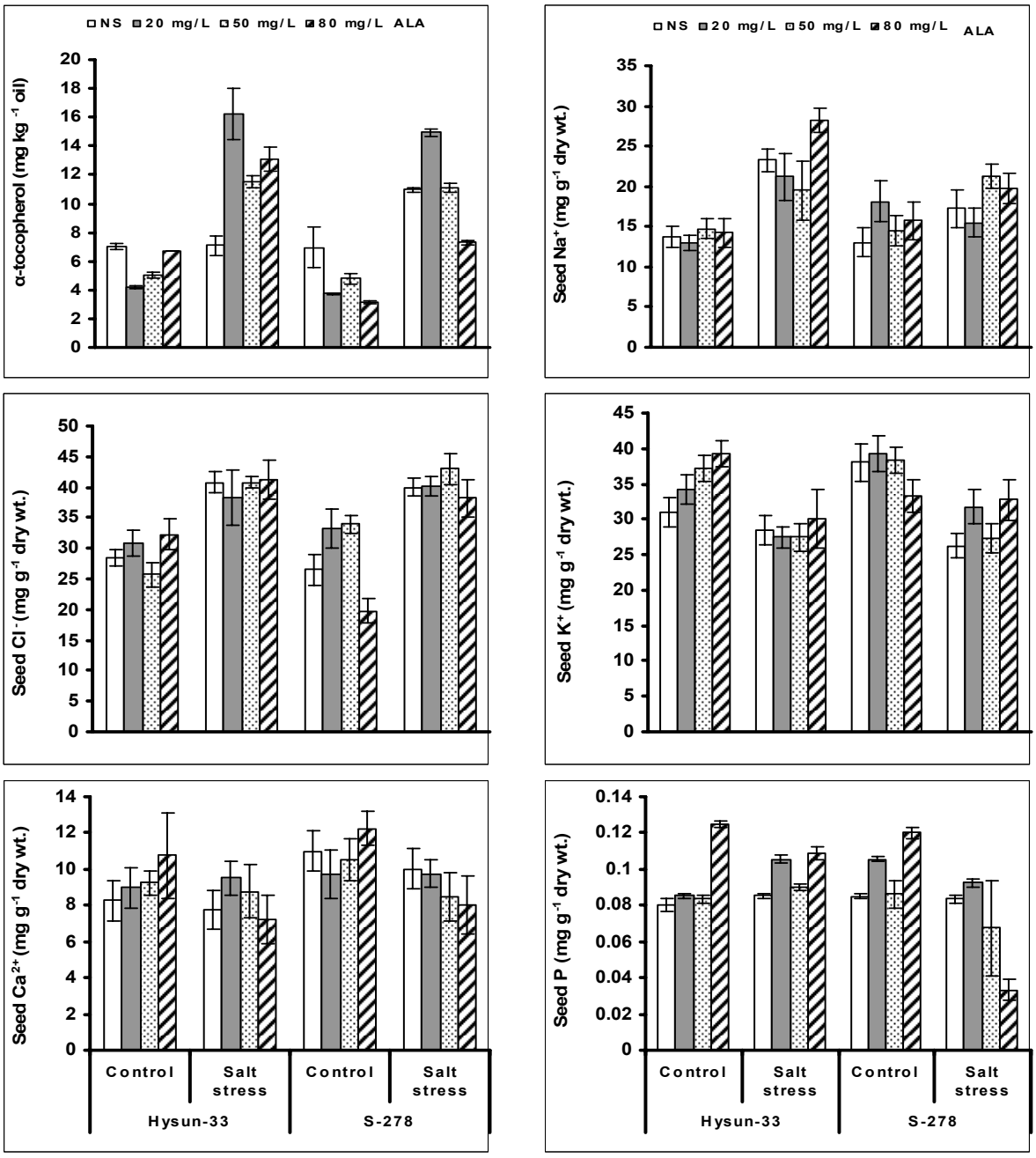

Fig. 2. α-tocopherols, Na^+, K^+, Cl^-, Ca^{2+} and P contents in seed oil of salt-stressed and non-stressed plants of two cultivars of sunflower (*Helianthus annuus* L.) subjected to foliar-applied varying levels of 5-aminolevulinic acid (Mean ± S.E.). NS stands for No Spray.

Pattern of accumulation of inorganic nutrients (P, K^+, Cl^-, Ca^{2+} and Na^+) under non-saline and saline regimes in sunflower seeds were recorded and presented in Table 1; Fig. 2). NaCl stress markedly enhanced the seed Cl^- and Na^+ accumulation, but, in contrast, a considerable suppression in seed K^+ and P concentrations was recorded, however, seed Ca^{2+} concentration remained unaffected under salt stress. Both sunflower cultivars were similar in seed Na^+, K^+, Ca^{2+} and Cl^-, while seed P was significantly ($p \leq 0.05$) lower in cv. S-278 as compared to that in the other sunflower cultivar. Exogenous application of different levels of ALA did not affect the inorganic nutrient concentrations in both sunflower cultivars, however, a significant ($p \leq 0.05$) improvement in seed P was found at 20 and 80 mg L^{-1} of ALA under non-saline regimes in both sunflower cultivars, while under saline conditions, only a significant improvement was observed in cv. Hysun-33 at 20 and 80 mg L^{-1}. However, in contrast, a considerable reduction in seed P was observed at 50 and 80 mg L^{-1} in cv. S-278 under saline regimes (Fig. 2).

Discussion

In the present study, salt stress had a negative effect on yield and yield related attributes of both sunflower lines. Our results support the results of some earlier reports in which salt-induced inhibition in seed yield and yield components have been reported in different plant species e.g. sunflower (Akram & Ashraf, 2011), mungbean (Ahmed, 2009), wheat (Shahbaz *et al.,* 2008), and barley (Endris & Mohammed, 2007). Generally, the salt-induced inhibition in yield is associated to decline in chlorophyll contents, stomatal and non-stomatal limitations for gas exchange, high level of Cl^- and Na^+ in plant tissues, oxidative damage (Ashraf, 2009; Khan *et al.,* 2009; Akram & Ashraf, 2011). Although exogenous application of ALA is known to enhance yield in different plants (Roy & Vivekanandan, 1998; Al-Khateeb *et al.,* 2006; Al-Thabet, 2006; Zhang *et al*., 2006), our results presented here are otherwise i.e., no marked effect of ALA application was observed on seed yield of the sunflower plants of both cultivars examined in the study.

Nutrient imbalance in plant tissues is one of the vital salt-induced adversaries (Munns *et al.,* 2006; Akram & Ashraf, 2011). In the present study, salt stress considerably enhanced the seed Cl^- and Na^+ accumulation, while considerably suppressed that of K^+ and P. Exogenous application of different levels of ALA did not affect the inorganic nutrient concentrations in both sunflower cultivars, except a significant improvement in seed P was found at 20 and 80 mg L^{-1} of ALA under saline regimes. Recently, Naeem *et al.* (2010) have shown that foliar applied ALA enhanced accumulation of various micro- and macro-nutrients such as, Fe, P, K, Mg, Ca, S, and N in oilseed rape leaf tissues. However, in the present study, foliar-applied ALA altered only seed P. Recently, seed and fruit Na^+, Cl^- and K^+ concentrations and their association with the germination capacity of *Crithmum maritimum* was determined. The fruits accumulated 8-11-fold more Na^+ and Cl^- than did the seeds. In contrast, the seeds maintained significantly higher K^+/Na^+ ratio than did the fruits under saline stress (Atia *et al.*, 2010).

Seed oil percentage of both sunflower cultivars decreased under saline conditions and almost a similar trend of decrease in seed oil contents (%) was found in both cultivars. Salt stress changed the quantity and quality of seed oil of different oil-seed crops (Raju & Ranganayakulu, 1978; Noreen & Ashraf, 2010). A considerable salt-induced decrease in seed oil was found in different oil-seed crops such as safflower (Bassil & Kaffka, 2002; Siddiqi, 2010), sunflower (Noreen & Ashraf, 2010), stock (*Matthiola tricuspidata*), rapeseed (Heuer *et al.,* 2002), and olive (Zarrouk *et al.,* 1996). In the present study, a significant alteration in oil contents of both sunflower cultivars was found as a result of foliar-applied 50 and 80 mg L^{-1} ALA under saline as well as non-saline conditions. These findings should be considered as the first report as not a single report on ALA-induced changes in seed oil contents can be found from the literature. So, detailed studies are required to be carried out to elucidate the role of ALA on seed oil contents in different oil-seed crops.

Tocopherols, also known as vitamin E, play a significant role in maintaining the oil quality by counteracting the stress-induced oxidative stress by acting as important non-enzymatic antioxidants. However, their concentration varies greatly among different oil-seed crops (Abbasi *et al.,* 2007; Noreen & Ashraf, 2010; Siddiqi, 2010). In the present investigation, salt stress caused enhanced accumulation of α-tocopherols in both sunflower cultivars. It is now evident from some previous studies that seed-oil tocopherols are altered under stress conditions. For example, Siddiqi (2010) reported that a considerable increase in Δ-, α- as well as total tocopherol contents occurred in safflower seed-oil on exposure of plants to salt stress. Similarly, Noreen & Ashraf (2010) observed an increase in α-tocopherols in sunflower seed-oil, while in contrast, Anwar *et al.* (2006) observed a considerable reduction in α-tocopherols in *Moringa oleifera* under saline conditions. In the present study, high biomass producing sunflower cultivar, Hysun-33 was relatively higher in α-tocopherols than the low biomass producing cultivar S-278. Foliar-applied 20 mg L^{-1} ALA significantly enhanced the accumulation of α-tocopherols in both sunflower cultivars under saline conditions, but such findings cannot be deciphered from the existing literature.

In this investigation, of various physical characteristics of sunflower seed-oil, moisture contents of the oil were adversely affected due to saline regimes. In contrast, salt stress or ALA had no significant effect on refractive index, seed organic as well as seed inorganic contents. Salinity has been reported to have no effect on the physical characteristics of seed-oil of different crops such as cotton (Ahmad *et al.,* 2007), *Moringa oleifera* (Anwar *et al.,* 2006), sunflower (Noreen & Ashraf, 2010), and ajwain (Ashraf & Orooj, 2006). However, non-significant effect of ALA in both sunflower cultivars cannot be explained because not a single report on ALA-induced changes in seed oil characteristics was found from the literature.

In conclusion, salt stress adversely affected the achenes/plant, total achene yield, 100-achene weight, seed moisture content, seed oil percentage, seed K^+ and P concentrations of both sunflower cultivars, while in contrast, salt-induced increase was observed in the

accumulation of seed Cl^- and Na^+ as well as α-tocopherols in the oil of both sunflower cultivars. Foliar-applied varying levels of ALA remained ineffective in improving all the yield variables, oil refractive index and seed inorganic nutrients (Na^+, Cl^-, K^+ and P) of both sunflower cultivars under control and saline regimes. However, a significant increase in seed oil concentration, accumulation of oil α-tocopherols and seed P of both sunflower cultivars was observed due to foliar-applied ALA under saline conditions. Overall, ALA was ineffective in ameliorating the salt-induced adverse effects on yield and different seed-oil characteristics except of seed P and oil α-tocopherols in sunflower plants.

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