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Improved salt tolerance by α-tocopherol in soybean involves up-regulation of ascorbate-glutathione cycle and secondary metabolites

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Summary

The effects of α-tocopherol on growth, photosynthesis, oxidative parameters, and tolerance mechanisms in soybean under increased salinity were studied. Salinity stress reduced shoot length, dry weight, chlorophyll and carotenoids, photosynthesis, and PSII activity; however, α-tocopherol mitigated the decline considerably. Salinity stress caused accumulation of superoxide ($O_2^{\cdot-}$) hydrogen peroxide (H_2O_2), thereby increased lipid peroxidation and decreased membrane stability index. Application of α-tocopherol ameliorated oxidative damage by reducing lipid peroxidation and downregulating lipoxygenase activity. The up-regulation of antioxidant and glyoxylase systems protected soybean from the damaging effects of ROS and methylglyoxal. Moreover the content of ascorbate, reduced glutathione and α-tocopherol increased significantly. The activity of γ-glutamyl kinase also increased due to application of α-tocopherol and accumulation of Na^+ was significantly declined with enhancement in K^+ uptake. Therefore results of present study revealed the beneficial effect of foliar application of α-tocopherol under salinity stress in soybean.

Keywords: Glyoxylase system; Antioxidants; Osmolytes; Salinity; α-Tocopherol; Soybean.

Introduction

Salinity stress is considered as one of the damaging environmental factors responsible for significant decline in yield productivity (Kamran et al., 2020). Globally 20% of the agricultural land area is saline affected, thereby have serious impact on the growth and development of existing crops, and in future, it is expected that 50% of the arable land will be salinized by 2050 (Jamil et al., 2011). The less efficient management practices like excessive use of chemical fertilizers, polluted water for irrigation etc. have further aggravated the situation thus converting fertile, productive agricultural lands into less productive or wastelands (Machado and Serralheiro, 2017; Machado et al., 2017). Therefore it is imperative to have effective methods to counteract the damaging effects on the growth and developmental processes of plants growing on such soils (Soliman et al., 2020).

Salinity stress alters the growth and development of plants by declining root growth, mineral uptake and photosynthesis (Elkelish et al., 2019a). Salinity-induced excess generation of reactive oxygen species (ROS) results in oxidative damage to key macromolecules like proteins, lipids and nucleic acids (Ahmad et al., 2018). Excess ROS adversely affects the membrane functioning thereby impart damaging effects on the performance of key cellular organelles like chloroplasts and mitochondria (Ahanger et al., 2019). To counteract the damaging effects of excess salinity, plants upregulate the indigenously occurring tolerance mechanisms, which include (a) antioxidant system, (b) osmolyte accumulation, and (c) secondary metabolite accumulation (Umar et al., 2011; Sakhno et al., 2019). The antioxidant system is comprised of enzymatic and non-enzymatic components that work co-ordinately to prevent oxidative damage (Sakhno et al., 2019), while as osmolytes have an important role in tissue water relations (Soliman et al., 2019, 2020), besides their hand in oxidative stress mitigation. The proline accumulation under salt stress in plants is one of the striking responses (Szabados and Savouré, 2010; Fariduddin, et al., 2019). Proline is considered as a low molecular weight cyclic imino acid that provides osmotic adjustments (Kaur and Asthir, 2015), reduces the negative impacts of various reactive oxygen species (ROS) by modulating plants' antioxidant system (Naliwajski and Skłodowska, 2021; Stefanov et al., 2021). In addition proline also up-regulates the expression of genes under abiotic stress conditions (Nounjan et al., 2012; Hasanuzzaman et al., 2014; Teh et al., 2015; Arabia et al., 2021). Enzymes like pyrroline-5-carboxylate reductase and γ-glutamyl kinase are proline biosynthesis enzymes and under stress conditions their activities are increased along with the proline content (Meena et al., 2019). In an experiment to study the changes in plant growth parameters and proline metabolism in *Rauwolfia serpentina* under salinity stress, Misra and Misra (2012) have observed that pyrroline-5-carboxylate reductase as well as γ-glutamyl kinase were up-regulated in contrast to down-regulation of proline oxidase activity which together contributed to increased proline content. Serefliogu et al. (2017) studied the effect of α-tocopherol in mediating salt stress tolerance in soybean. The results suggested the involvement of auxin biosynthesis rather than enhancement antioxidant defense was more effective in countering the salt stress damages. The up-regulation of tolerance mechanisms has been reported to lessen the damaging effects of stresses on germination, growth, photosynthesis, mineral uptake, and assimilation, hence yield (Ahanger and Agarwal, 2017).

Tocopherols and tocotrienols are lipid-soluble molecules belonging to vitamin E group compounds and are combinedly known as tocochromanols and are synthesized only by photosynthetic organisms. The detailed characterization of mutants and transgenic plants exhibiting altered tocopherol synthesis have confirmed its beneficial role in plant growth and development (reviewed by Falk and Munne-Bosch, 2010). Plants exhibiting deficient tocopherol synthesis exhibit declined germination, photoassimilate export and growth, increased leaf senescence, and altered responses to stresses (Ellouzi et al., 2013; Allu et al., 2017). Tocopherols scavenge lipid peroxy radicals preventing lipid peroxidation, besides, protect lipids and membranes by physically quenching or chemically reacting with singlet oxygen (Munne-Bosch and Alegre, 2010). In addition, α-tocopherol can mediate signaling, through direct or indirect interaction with key signaling components (Rimbach et al., 2002; Munne-Bosch and Alegre, 2010; Zingg, 2019), and can also bring modulation of signal transduction pathways (Zingg, 2019) as well as phytohormone levels (Serefliogu et al., 2017).

Soybean (*Glycine max* L.) is an important legume crop widely grown for edible bean and is consumed worldwide as a vegetable; besides soy milk, tofu and tofu skin are made from it. It is very rich in proteins and other important growth-promoting ingredients like anthocyanins (Harlen and Jati, 2018). The presence of some key compounds like isoflavones, coumestrol, phytate, saponins, lecithin,

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phytosterols, and dietary fibers make it appropriate for pharmacological purposes for the treatment of several diseases (BADOLE et al., 2015). Stresses, including salinity, drastically damage the quality and quality of soybean productivity all over the world. Therefore in the present study, it was hypothesized that exogenous application of α-tocopherol could ameliorate salinity triggered growth and photoinhibition by upregulating tolerance mechanisms like antioxidant system, osmolyte, and secondary metabolite metabolism.

Material and methods

Experimental design and treatment

Seeds of soybean (*Glycine max* L.) were disinfected by dipping into 0.001% $HgCl_2$ for 5 min; therefore, seeds were washed with distilled water. Thereafter seeds were sown in earthen pots filled with garden soil and compost in the ratio of 3:1. Pots were regularly monitored and maintained under the greenhouse and were arranged in a complete randomized block design with five replicates for each treatment. At the time of sowing, all pots were wetted by applying 300 mL full-strength Hoagland nutrient solution, and details of the Hoagland nutrient solution used as: The composition of nutrient solution used was 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 1 mM $NH_4H_3PO_4$, 50 μM KCl, 25 μM H_3BO_4, 2 μM $MnCl_2$, 20 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM $(NH_4)6Mo_7O_{24}$, and 20 μM Na_2Fe-EDTA (ELKELISH et al., 2019b). Ten days after germination, the number of seedlings was thinned to three per pot and were fed with 200 mL nutrient solution on alternate days for another 10 days. After 20 days of growth, pots were separated into two groups as (a) one group was irrigated with normal nutrient solution and (b) the second group was irrigated with nutrient solution containing 100 mM NaCl. To one group of pots, foliar application of α-tocopherol (15 mL per pot) at 100 and 500 μM was given twice a week using a sprayer. Detailed treatments in present experiment included: (a) control (nutrient solution), (b) 100 μM tocopherol (TOP), (c) 500 μM tocopherol, (d) 100 mM NaCl (e) 100 mM NaCl + 100 μM tocopherol, and (f) 100 mM NaCl + 500 μM tocopherol. Thirty-five days old seedlings were uprooted and analysed for different parameters, including oxidative stress parameters, osmolytes, secondary metabolites, antioxidants and glyoxalase system.

Plant height and dry weight

The plant height was measured using a manual scale however, for estimation of dry weight, plants were dried in an oven at for 48 h at 70 °C.

Estimation of photosynthetic pigments, photosynthesis, and photochemical efficiency

The content of total chlorophylls and carotenoids was determined according to ARNON (1949). Fresh 100 mg leaf tissue was extracted in 80% acetone using pestle and mortar. After centrifugation, the absorbance of the supernatant was measured at 480, 645, and 663 nm.
For measuring the rate of net photosynthesis (*Pn*) a portable infrared gas analyzer (CID-340, Photosynthesis System, Bio-Science, USA) was employed. Maximal photochemical efficiency (Fv/Fm) was determined using modulated chlorophyll fluorometer (PAM 2500; Walz, Germany), and leaves were dark-adapted for 25 min.

Estimation of leaf relative water content, sugars, and glycine betaine

For measurement of leaf relative water content (LRWC), leaf discs were punched from fresh leaves, and their fresh weight was recorded. Same discs were floated on water in Petri dishes to get turgid weight, followed by their drying in an oven at 80 °C for 24 h for recording the dry weight (SMART and BINGHAM, 1974). The calculation was done using the following formula:

$$RWC = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100$$

For estimation of sugar content, the method described by SCHIELDS and BURNET (1960) was adopted. Briefly, a 500 mg dry powdered sample was extracted in 80% ethanol, and the homogenate was centrifuged for 20 min at 5000 × *g*. After that, the supernatant was collected and reacted with anthrone reagent, and optical density was recorded at 585 nm.The content of glycine betaine (GB) was estimated by extracting a dry powdered sample in distilled water. After that, the extract was mixed with H_2SO_4 (2N), and a cold KI-I_2 reagent was added. The resulting mixture was subjected to centrifugation at 10,000 × *g* for 15 min. After aspirating the supernatant, periodide crystals were dissolved in 1,2-dichloroethane, and optical density was recorded at 365 nm. The standard curve of GB was used for calculation (GRIEVE and GRATTAN, 1983).

Estimation of proline and assay of γ-glutamyl kinase

Proline content was estimated by homogenizing dried 500 mg sample in 3% sulphosalicylic acid. After centrifugation at 3000 × *g* for 20 min, 2 mL supernatant was mixed with 2 mL of each glacial acetic acid and ninhydrin reagent, and the mixture was incubated for 1h in a water bath at 100 °C. The reaction was terminated by keeping tubes on an ice bath, and proline was separated using toluene. Optical density was recorded at 520 nm (BATES et al., 1973).
The activity of γ-glutamyl kinase (GK, EC 2.7.2.11) was assayed by macerating fresh 500 mg leaf tissue in Tris buffer (pH 7.5) using cold pestle and mortar. The homogenate was centrifuged at 30,000 × *g* for 30 min, and the activity of γ-GK was determined in pellet following HAYZER and LEISINGER (1980). Reaction mixture contained 50 mM Tris buffer (pH 7.0), 50 mM L-glutamate, 20 mM $MgCl_2$, 10 mM ATP, 100 mM hydroxamate–HCl and reaction was initiated by addition enzyme. The reaction was terminated by the addition of stop buffer ($FeCl_3$ and TCA) and absorbance was taken at 535 nm, and amount (μg) of γ-glutamyl hydroxamate formed was considered as the activity of γ-GK and expressed as U mg^{-1} protein min^{-1}.

Measurement of membrane stability index and lipid peroxidation

The method described by SAIRAM et al. (1997) was used for measuring the membrane stability index (MSI), and calculation was done using the following formula:

$$MSI = [1 - (C_1/C_2)] \times 100$$

For determination of lipid peroxidation, content of malonaldehyde (MDA) formation according to HEATH and PACKER (1968) method was measured. Fresh 100 mg was homogenized in 1% trichloro acetic acid (TCA) using pestle and mortar. After centrifuging the extract at 10,000 × *g*, 1.0 mL supernatant was mixed with 0.5% thiobarbituric acid and heated at 95 °C for 1 h. After cooling the samples on an ice bath and centrifuged again for 5 min at 5000 × *g*. The optical density of the supernatantwas recorded at 532 and 600 nm.

Estimation of hydrogen peroxide and superoxide, and activity of lipoxygenase

Hydrogen peroxide was determined according to the method of VELIKOVA et al. (2000). Briefly, 100 mg tissue was homogenized in 0.1% TCA, and the homogenate was centrifuged for 15 min at 12,000 × *g*. To 0.5 mL supernatant was added 0.5 mL of potassium phosphate buffer (pH 7.0) and 1 mL of potassium iodide followed by measurement of absorbance at 390 nm. For determination of super-

oxide, fresh tissue was extracted in potassium phosphate buffer (65 mM, pH 7.8), and the homogenate was centrifuged at 5000 × *g* for 10 min. The supernatant was mixed with 10 mM hydroxylamine hydrochloride and left for 20 min followed by the addition of sulfanilamide and naphthylamine, and the resulting mixture was incubated at 25 °C for 20 min. Thereafter optical density was recorded at 530 nm, and calculations were done using a standard graph of $NaNO_2$ (Yang et al. 2011).

Lipoxygenase (LOX, EC 1.13.11.12) activity was assayed following Doderer et al. (1992), and an increase in absorbance was recorded at 234 nm using linoleic acid as substrate. For calculation extinction coefficient of 25 mM^{-1} cm^{-1} was used, and activity was expressed as units mg^{-1} protein equivalent to 1 μmol of substrate oxidized min^{-1}.

Determination of glyoxalase I and II activity and methylglyoxal (MG) content

Extraction of glyoxylase I (EC: 4.4.1.5) and glyoxalase II (EC: 3.1.2.6) was carried by homogenizing fresh 500 mg tissue in cold potassium phosphate buffer (50 mM; pH 7.0) supplemented with KCl (10 mM), ascorbate (1 mM), β-mercaptoethanol (5 mM) and glycerol (10%). After centrifuging the homogenate for 15 min at 11,500 × *g*, the supernatant was collected and used as an enzyme source. The activity of Gly I was assayed following Hasanuzzaman et al. (2011), and change in optical density was monitored at 240 nm in an assay mixture containing GSH (100 mM), 0.1 M phosphate buffer, $MgSO_4$ (16 mM), methylglyoxal (35 mM) and enzyme extract. The extinction coefficient of 3.37 mM^{-1} cm^{-1} was used for calculation, and activity was expressed as μmol min^{-1} mg^{-1} protein. Gly II activity was determined in 1 mL assay mixture containing Tris-HCl buffer (100 mM, pH 7.2), DTNB (0.2 mM), and *S*-D-lactoylglutathione (1 mM). Optical density was recorded at 412 nm, and calculation was done using an extinction coefficient of 13.6 mM^{-1} cm^{-1} (Principato et al., 1987).

The content of MG in leaves was determined by homogenizing leaf tissue in perchloric acid (5%). After centrifuging the homogenate for 10 min at 11,000 × *g*, supernatant was decolorized by charcoal, followed by the addition of sodium dihydrogen phosphate and N-acetyl-L-cysteine. After 10 min, the N-α-acetyl-*S*-(1-hydroxy-2-oxo-prop-1-yl) formed was read at 288 nm (Wild et al., 2012).

Determination of activities antioxidant enzymes

For extraction of antioxidant enzymes 1 gm fresh leaf tissue in cold 50 mM phosphate buffer (pH 7.0) containing 1% polyvinyl pyrolidine and 1 mM EDTA using pre-chilled pestle and mortar. Homogenate was centrifuged at 15,000 × *g* for 20 min at 4 °C and supernatant was collected and used as enzyme source. Protein was estimated according to Lowry et al. (1951).

Activity of superoxide dismutase (SOD, EC 1.15.1.1) was determined according to method of Bayer and Fridovich (1987) by recording the photochemical reduction of NBT at 560 nm. The assay mixture contained 50 mM sodium phosphate buffer (pH 7.5), 100 μL of EDTA, L-methionine, 75 μM of NBT, riboflavin, and 100 μL enzyme extract in a final volume of 1.5 mL. The assay mixture was incubated for 15 min, and the reaction was terminated by switching off the light, and activity was expressed as EU mg^{-1} protein. The ascorbate peroxidase activity (APX, EC 1.11.1.11) was determined by monitoring the change in absorbance at 290 nm for 3 min. The assay mixture contained potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, hydrogen peroxide, and enzyme extract in the final volume of 1 mL. An extinction coefficient of 2.8 mM^{-1} cm^{-1} was used for calculation (Nakano and Asada, 1981). For assaying the activity of glutathione reductase (GR; EC 1.6.4.2) was assayed according to the method of Foyer and Halliwell (1976). Change in absorbance was recorded at 340 nm for 2 min in a reaction mixture containing sodium phosphate buffer (pH 7.8), 0.1 mM nicotinamide adenine dinucleotide phosphate (NADPH), 0.5 mM oxidized glutathione (GSSG), and enzyme extract. For calculation extinction coefficient of 6.2 mM^{-1} cm^{-1} was used.

Estimation of ascorbate, reduced glutathione, and tocopherol

For estimating the content of ascorbate (AsA) method described by Mukherjee and Choudhuri (1983) was employed. Briefly, fresh plant material was macerated in 6% TCA using pestle and mortar, and the homogenate was centrifuged at 5000 × *g* for 10 min. The supernatant was mixed with 2% dinitrophenylhydrazine and 10% thiourea. Mixture was boiled for 15 min in a water bath, and after cooling, 5 mL of cooled 80% H_2SO_4 was added and absorbance recorded at 530 nm. For calculation standard curve of ascorbate was used.

Content of reduced glutathione (GSH) was estimated by macerating fresh tissue in phosphate buffer (pH 8.0) followed by centrifugation at 3000 × *g* for 15 min. To 0.5 mL supernatant was added 0.5 mL of 5,5-dithiobis-2-nitrobenzoic acid and left for 10 min. Thereafter absorbance was recorded at 412 nm, and the content of GSH was calculated from a standard curve of GSH (Ellman, 1959).

Tocopherol was extracted in ethanol and petroleum ether in the ratio of 1.6:2. Thereafter supernatant was reacted with 2% of 2,2-dipyridyl and incubated in dark, followed by measurement of optical density at 520 nm (Backer et al., 1980). A standard curve of α-tocopherol was used for calculation.

Determination of phenols, flavonoids, and total antioxidant

For estimation of total phenols 500 mg dry powdered sample was extracted in methanol. After centrifugation at 10,000 × *g* for 10 min, the supernatant was reacted with Folin-Ciocalteu reagent and optical density was recorded at 765 nm (Singleton and Rossi, 1965). For calculation standard curve of gallic acid was used. The content of flavonoids was determined following the method of Zhishen et al. (1999). After extracting the tissue in methanol supernatant was reacted with $NaNO_2$ and $AlCl_3$ followed by the addition of 2 mL NaOH and 2.4 mL distilled water. Absorbance was taken at 510 nm, and a standard curve of catechin was used for calculation. Total antioxidant activity was determined by extracting the leaf tissue in methanol and centrifuging the homogenate at 10,000 g for 10 min. Thereafter supernatant was reacted with 1,1-dipheny l-2-picrylhydrazyl (DPPH, 0.1 mM) and incubated for 30 min in the dark. Optical density was recorded at 517 nm (Shimada et al., 1992).

Estimation of Na^+ and K^+

Dried leaf (1.0 g) tissue was digested in a mixture of $HClO_4$ and HNO_3 until the solution turned clear. Digested material was made to 100 mL by adding distilled water and read on flame photometer for quantification of Na^+ and K^+ (Wolf, 1982).

Statistical analysis

Data is the mean (±SE) of five replicates. The least significant difference (LSD) at $p < 0.05$ was calculated using Duncan's Multiple Range Test.

Results

Application of tocopherol improves the height, dry weight, and RWC

The results showing the effects of exogenous tocopherol on height and dry weight under normal and salt stress conditions are present-

ed in Tab. 1. Related to control, height decline by 34.34% and dry weight by 37.26% due to salt stress; however, application of tocopherol increased both parameters at both concentrations with a maximal increase of 27.50% and 40.79% exhibited by seedlings treated with 500 µM tocopherol (Tab. 1). Application of 500 µM tocopherol to NaCl stressed soybean seedlings resulted in maximal amelioration. Salinity declined LRWC by 32.36% over control, however, was enhanced by 2.46% and 5.99% due to the application of 100 and 500 µM α-tocopherol, respectively. Relative to NaCl stressed plants, the percent increase in LRWC was 9.81% in NaCl + 100 µM α-tocopherol and 22.42% in NaCl + 500 µM α-tocopherol treated seedlings depicting mitigation of decline (Tab. 1).

Effect of α-tocopherolon Na^+ and K^+ content

Salinity stress increased (179.29%) Na^+ accumulation with a significant decline (50.24%) in K over control. Application of α-tocopherol reduced Na^+ content by 9.84% and 59.59% at 100 and 500 µM concentrations. Related to NaCl stressed seedlings, application of α-tocopherol declined the content of Na^+ by 21.97% and 44.30%, respectively, in NaCl + 100 µM α-tocopherol and NaCl + 500 µM α-tocopherol treated seedlings. However, decline in K^+ was mitigated significantly due to the application of tocopherol (Tab. 1).

Exogenous tocopherol application enhances osmolyte accumulation and γ-glutamyl kinase activity

Salinity stressed seedlings exhibited enhanced accumulation of compatible osmolytes, including sugars, glycine betaine, and proline (Fig. 1A-C). However, the application of tocopherol further enhanced their accumulation significantly. Maximal enhancement of 56.89% for sugars, 130.21% for glycine betaine, and 144.91% for proline was observed in seedlings treated with NaCl + 500 µM tocopherol. Under normal growth conditions, the increase in the content of sugars, GB, and proline was 29.70, 17.98, and 32.33%, respectively due to 500 µM tocopherol over control (Fig. 1A-C). The activity of γ-glutamyl kinase increased significantly due to application of tocopherol under normal and NaCl stress. Relative to control increase was 67.65% in salt-stressed seedlings which further increased by 22.24 and 60.10% due to application of 100 and 500 µM α-tocopherol over the NaCl treated counterparts. Under normal conditions, maximal enhancement in activity was observed due to 500 µM α-tocopherol (Fig. 1D).

Application of α-tocopherol reduces oxidative damage

Oxidative damage results measured in terms of MSI, MDA content, $O_2^{\cdot-}$ and H_2O_2 are shown in Tab. 2. It was observed that NaCl

Tab. 1: Effect of salinity (100 mM NaCl) with and without exogenous application of α-tocopherol (100 and 500 µM) on shoot length (cm), shoot dry weight (g/plant), leaf relative water content (LRWC), sodium and potassium (mg g^{-1} DW; DW = dry weight) content in soybean (*Glycine max*). Values are mean (±SE) of five replicates and different letters represent significant difference at $p<0.05$.

	Control	NaCl	100 µM α-tocopherol	500 µM α-tocopherol	NaCl + 100 µM α-tocopherol	NaCl + 500 µM α-tocopherol
Shoot length (cm)	1.20 ± 0.115b	0.7879 ± 0.039e	1.29 ± 0.101b	1.53 ± 0.068a	0.814 ± 0.023d	0.870 ± 0.019c
Shoot dry weight (g/plant)	6.52 ± 0.212b	4.09 ± 0.182e	6.82 ± 0.261b	9.18 ± 0.501a	4.79 ± 0.128d	5.85 ± 0.201c
LRWC (percent)	82.40 ± 4.65b	55.73 ± 2.92e	84.43 ± 4.54b	87.34 ± 4.14a	61.20 ± 3.32d	68.23 ± 3.1c
Na^+ (mg g^{-1}DW)	3.96 ± 0.37d	11.06 ± 0.60a	3.57 ± 0.31d	1.60 ± 0.36e	8.63 ± 0.55b	6.16 ± 0.50c
K^+ (mg g^{-1} DW)	14.13 ± 0.97c	7.03 ± 0.40e	15.00 ± 1.12b	19.87 ± 1.24a	8.26 ± 0.65	13.06 ± 1.76d

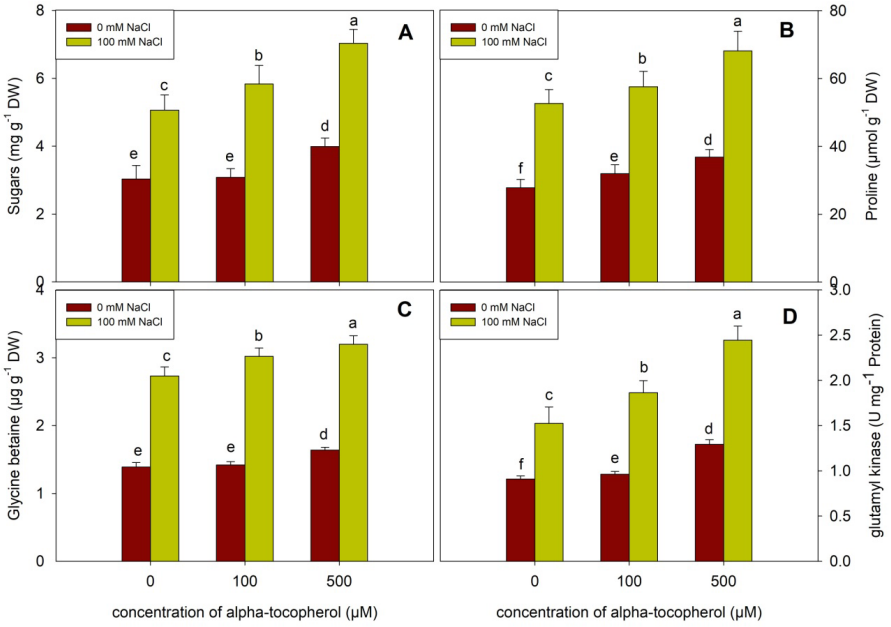

Fig. 1: Effect of increased salinity (100 mM NaCl) stress with and without exogenous application of α-tocopherol (100 and 500 µM) on (A) sugars, (B) proline, (C) glycine betaine and (D) γ-glutamyl kinase activity in soybean (*Glycine max*). Values are mean (±SE) of five replicates and different letters represent significant difference at $P<0.05$.

Tab. 2: Effect of salinity (100 mM NaCl) with and without exogenous application of α-tocopherol (100 and 500 µM) on superoxide, hydrogen peroxide, membrane stability index, lipid peroxidation, lipoxygenase activity, total phenols, total flavonoids and total antioxidant activity (DPPH) in soybean (*Glycine max*). Values are mean (±SE) of five replicates and different letters represent significant difference at P<0.05.

	Control	NaCl	100 µM α-tocopherol	500 µM α-tocopherol	NaCl + 100 µM α-tocopherol	NaCl + 500 µM α-tocopherol
Superoxide (nmol g^{-1} FW)	9.1 ± 0.750d	21.2 ± 2.05a	7.50 ± 0.580e	5.7 ± 0.58f	17.6 ± 0.51b	14.0 ± 0.75c
Hydrogen peroxide (nmol g^{-1} FW)	13.06 ± 1.50d	25.29 ± 3.405a	10.96 ± 0.907e	7.46 ± 0.723f	20.23 ± 1.46b	17.36 ± 1.234c
Membrane stability index (Percent)	83.50 ± 3.45b	55.90 ± 2.30e	84.20 ± 4.82b	89.61 ± 3.41a	58.14 ± 2.99d	67.64 ± 2.99c
Lipid peroxidation (mol g^{-1} FW)	25.66 ± 2.80d	42.03 ± 4.11a	20.6 ± 2.60e	14.5 ± 1.8f	37.96 ± 3.66b	31.09 ± 2.72c
Lipoxygenase (µmol min^{-1} mg^{-1}protein)	13.88 ± 0.67d	28.81 ± 2.100a	11.89 ± 0.627e	7.89 ± 0.401f	23.12 ± 1.61b	16.89 ± 0.92c
Total phenols (mg g^{-1} DW)	3.06 ± 0.152d	3.166 ± 0.251c	3.263 ± 0.118c	3.866 ± 0.152a	3.146 ± 0.305c	3.543 ± 0.14b
Flavonoids (mg g^{-1} DW)	1.133 ± 0.066b	1.047 ± 0.115c	1.163 ± 0.094b	1.657 ± 0.084a	1.102 ± 0.060b	1.670 ± 0.19a
DPPH (percent)	56.13 ± 2.83d	57.4 ± 2.29d	64.30 ± 2.78c	71.8 ± 2.12b	74.36 ± 4.01b	79.63 ± 3.52a

stress resulted in increased accumulation of $O_2^{\cdot-}$ and H_2O_2 resulting in greater lipid peroxidation and declined MSI. Application of α-tocopherol at both concentrations reduced accumulation of $O_2^{\cdot-}$ and H_2O_2 with the maximal decline of 37.36% and 42.87% at 500 µM α-tocopherol, imparting a decline of 43.49% in lipid peroxidation over the control (Tab. 2). Relative to NaCl stressed seedlings, the concentration of $O_2^{\cdot-}$ and H_2O_2 declined by 16.98 and 20.00% in NaCl + 100 µM α-tocopherol and by 33.96 and 31.35% in NaCl + 500 µM α-tocopherol respectively, declining lipid peroxidation by 9.68% and 26.02% respectively (Tab. 2). Salinity declined MSI by 33.05%, while applying tocopherol at both concentrations concentration mitigated the decline significantly (Tab. 2). The activity of lipoxygenase increased (107.56%) by NaCl stress, however was declined due to the application of α-tocopherol. A maximal decline of 43.15% was observed in seedlings treated with 500 µM α-tocopherol (Tab. 2).

Effect of salinity and α-tocopherol on phenols, flavonoids and total antioxidant activity

The application of exogenous tocopherol resulted in the increased accumulation of phenols and flavonoids under normal and salinity stress conditions (Tab. 2). Relative to control, phenols and flavonoids maximally increased by 15.78% and 47.39% in 100 mM + 500 µM α-tocopherol treated seedlings. Under normal growth conditions content of phenols increased by 6.63% and 26.33% due to 100 and 500 µM α-tocopherol, while flavonoids increased by 2.64% and 46.24% due to 100 and 500 µM α-tocopherol (Tab. 2). Total antioxidant activity measured in terms of DPPH radical scavenging activity showed a significant enhancement in α-tocopherol treated seedlings with maximal improvement observed at 500 µM concentration under normal as well as NaCl stress conditions (Tab. 2). The DPPH scavenging increased maximally by 41.88% in NaCl + 500 µM α-tocopherol treated seedlings over the control (Tab. 2).

Effect of salinity and α-tocopherol on pigment synthesis, photosynthesis and PSII activity

Salinity stress resulted in the decline of total chlorophylls (39.08%), carotenoids (29.66%), photosynthesis (34.35%), and Fv/Fm (15.81%) over the control plants (Fig. 2A-D). Relative to NaCl stressed seedlings the decline in total chlorophylls, carotenoids, photosynthesis and Fv/Fm was maximally ameliorated by 37.76%, 32.33%, 38.67% and 32.15% respectively in NaCl + 500 µM α-tocopherol treated seedlings. Under normal conditions, application of α-tocopherol increased total chlorophylls, carotenoids, photosynthesis, and Fv/Fm, exhibiting a maximal increase of 42.35%, 53.41%, 34.42%, and 23.08% respectively at 500 µM α-tocopherol (Fig. 2A-D).

The activity of antioxidant enzymes and the content of non-enzymatic antioxidants

Soybean seedlings exposed to 100 mM NaCl exhibited an increase in the activity of SOD, APX and GR over the control (Fig. 3). Relative to control, the activity of SOD, APX and GR increased by both α-tocopherol concentrations; however, maximal enhancement of 24.22% for SOD, 15.60% for APX, and 20.35% for GR over control was observed due to 500 µM α-tocopherol. Relative to control, maximal enhancement in the activities of SOD (104.47%), APX (45.39%), and GR (111.50%) was observed in NaCl + 500 µM α-tocopherol treated seedlings (Fig. 3A-C). Relative to NaCl stressed plants application of 500 µM α-tocopherol further increased the activity of SOD by 32.69%, APX by 12.02% and GR by 26.45%. The contents of GSH, AsA, and α-tocopherol increased due to exogenously supplied α-tocopherol at both concentrations. Treatment of NaCl increased content of GSH (18.56%) and α-tocopherol (27.85%) while as decreased AsA (15.94%) over control (Fig. 4). Content of GSH and α-tocopherol further increased by exogenous application of α-tocopherol attaining maximal enhancement at 500 µM. Relative to NaCl stressed plants content of GSH and tocopherol was further increased by 11.10% and 129.5% respectively in NaCl + 500 M tocopherol treated plants while as the decline in AA was mitigated by 29.14%. However, the decline in AsA was mitigated by exogenously applied α-tocopherol (Fig. 4A-C).

Effect on the activity of Gly I and II, and content of MG

Salinity stress resulted in increased activity of Gly I and II by 18.79% and 6.92% over control; however, application of 500 µM α-tocopherol increased activity of Gly I and II by 4.51% and 3.98% at 100 µM α-tocopherol and by 47.36% and 56.34% at 500 µM α-tocopherol (Fig. 5A and B). Application of α-tocopherol to salini-

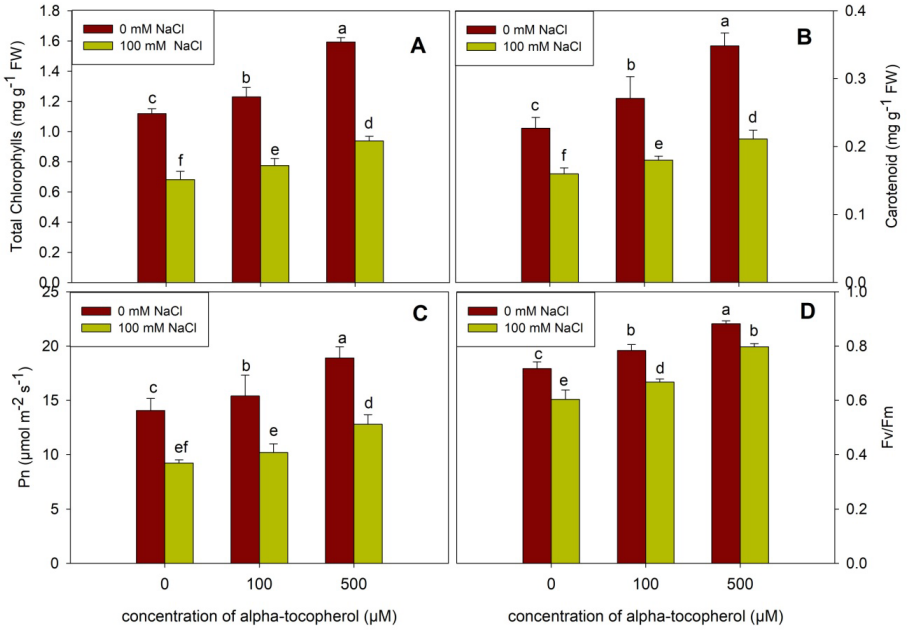

Fig. 2: Effect of increased salinity (100 mM NaCl) stress with and without exogenous application of α-tocopherol (100 and 500 µM) on (A) total chlorophylls, (B) carotenoids, (C) photosynthesis (Pn) and (D) PSII activity in soybean (*Glycine max*). Values are mean (±SE) of five replicates and different letters represent significant difference at P<0.05.

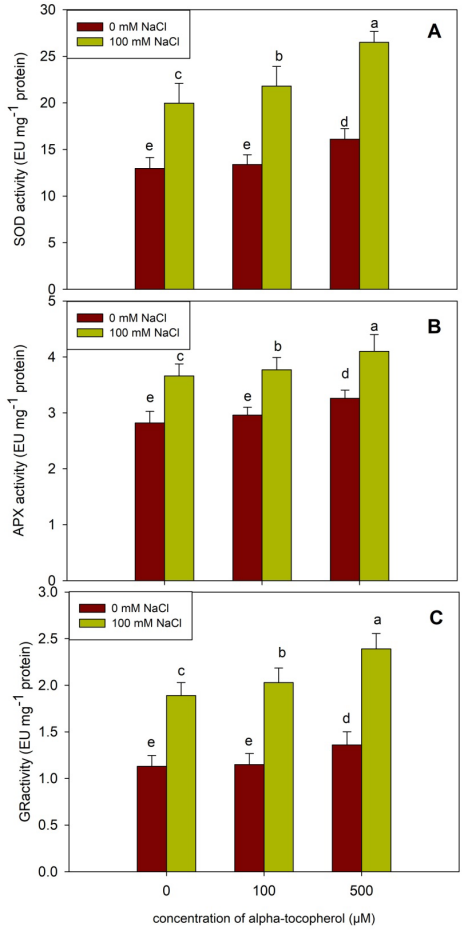

Fig. 3: Effect of increased salinity (100 mM NaCl) stress with and without exogenous application of-tocopherol (100 and 500 µM) on the activity of (A) superoxide dismutase (SOD), (B) ascorbate peroxidase (APX) and (C) glutathione reductase (GR) in soybean (*Glycine max*). Values are mean (±SE) of five replicates and different letters represent significant difference at P<0.05.

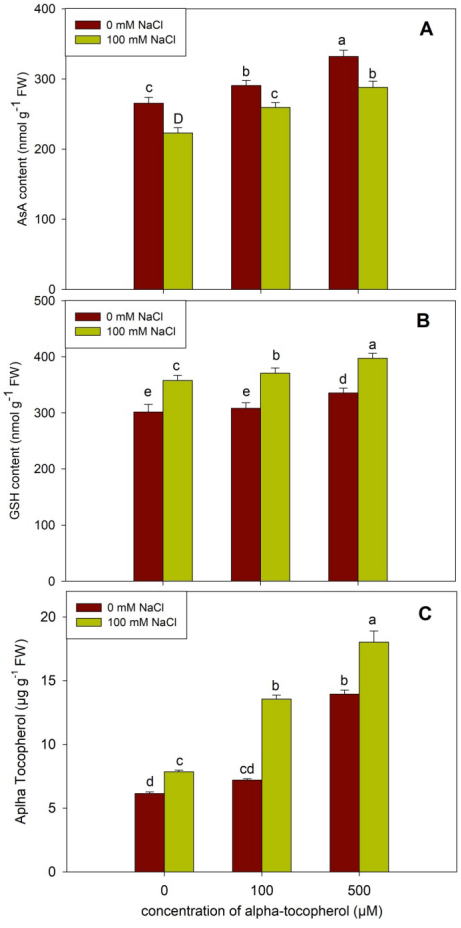

Fig. 4: Effect of increased salinity (100 mM NaCl) stress with and without exogenous application of α-tocopherol (100 and 500 µM) on (A) ascorbate, (B) reduced glutathione and (C) α-tocopherol content in soybean (*Glycine max*). Values are mean (±SE) of five replicates and different letters represent significant difference at P<0.05.

ty stressed seedlings further enhanced the activity of Gly I and II with a maximal increase of 111.27% and 87.93% due to NaCl + 500 µM α-tocopherol over NaCl stressed plants (Fig. 5A and B). Content of MG increased by 191.22% in NaCl stressed seedlings over control and exhibited a decline of 18.33% and 40.26% at 100 and 500 µM α-tocopherol (Fig. 5C). Application of α-tocopherol to salinity stressed seedlings reduced the accumulation of MG over the NaCl stressed counterparts with maximal reduction of 42.16% in NaCl + 500 µM α-tocopherol treated seedlings (Fig. 5C).

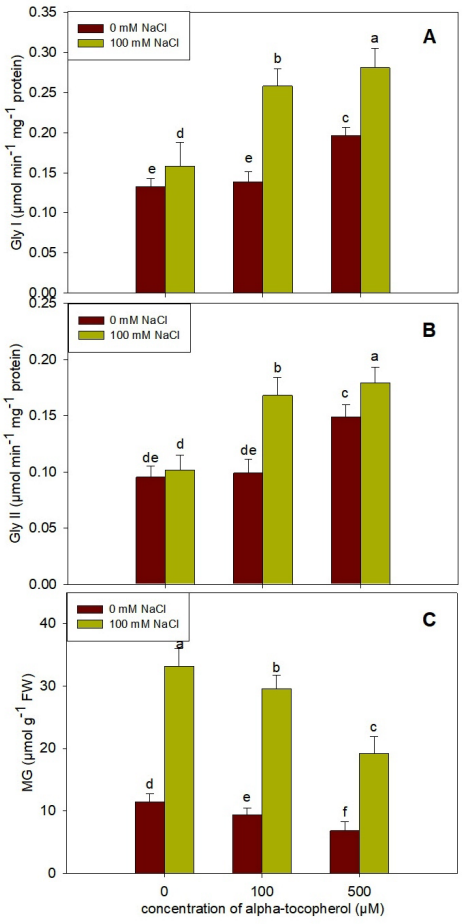

Fig. 5: Effect of increased salinity (100 mM NaCl) stress with and without exogenous application of α-tocopherol (100 and 500 µM) on the activity of (A) glyoxylase I, (B) glyoxylase II and (C) methylglyoxal content in soybean (*Glycine max*). Values are mean (±SE) of five replicates and different letters represent significant difference at P<0.05.

Discussion

Effect of salinity and α-tocopherol on growth, Na uptake and gene expression

Salinity is one of the damaging abiotic stress factors and several strategies have been devised to effectively lessen the damaging effects of high salinity and protect the yield potential. In the same direction, the present study was aimed to evaluate the role of the key antioxidant molecules in strengthening the salinity tolerance mechanisms in soybean. Salinity stress-mediated decline in growth and biomass production was obviously mitigated by applying α-tocopherol, with the effect being much obvious at higher concentrations. Declined growth and biomass production due to salinity has been observed by others as well (Ahmad et al., 2018; Elkelish et al., 2019a, b). Stresses hamper the cell division and cell cycle progression by affecting the expression of key genes (West et al., 2004; Qi and Zhang, 2019). Similar to our results application of tocopherol has been reported to improve the growth of *Vicia faba* under salinity stress (Semida et al., 2014). Treatment with tocopherol has earlier been reported to improve growth in soybean and the growth promotory effect can be attributed to increased synthesis of phytohormones like indole acetic acid (IAA) (Serefliogl u et al., 2017). Increased synthesis of tocopherol in transgenic *Arabidopsis thaliana* and alfalfa delays senescence (Jiang et al., 2016). The overexpression of *PDS1* gene in *A. thaliana* plants led to elevated tocopherol levels in leaves and seeds up to 43 and 28% respectively (Tsegaye et al., 2002). In another study,two *A. thaliana*q-hydroxyphenylpyruvate dioxygenase (*HPPD*) and 2-methyl-6-phytylplastoquinol methyltransferase (*MPBQ MT*) were over-expressed in corn kernel and it was found that in transgenic kernels as compared to their wild type counterparts up to 3 times more tocopherol was present (Naqvi et al., 2011). Transgenic *A. thaliana* deficit in tocopherol were more prone to P availability and photo-oxidative stress (Allu et al., 2017). In yet another classical study, Liu et al. (2008) found that transgenic tobacco plants over-expressing *tocopherol cyclase*(*VTE1*) from *A. thaliana*showed decreased lipid peroxidation, electrolyte leakage and H_2O_2 content, but increased chlorophyll under drought condition, which is in agreement with our present results. Kanwischer et al. (2005) reported that in transgenic and mutant plants of *A .thaliana* alterations in tocopherol cyclase activity affected the content of tocopherol content to a considerable level. Increased growth in soybean due to exogenous application of α-tocopherol in the present study can be ascribed mainly to reduced accumulation of Na^+. Increased accumulation of Na^+ is considered toxic to plant cellular metabolism as it interferes with enzyme functioning, imparts oxidative damage, and restricts root growth (Elkelish et al., 2019a; Arif et al., 2019). Besides, salinity influences the uptake of key minerals like N, P, K, etc., and their assimilation pathways (Ahanger and Agarwal, 2017), thereby affecting the growth and metabolism. However, α-tocopherol application declined Na^+ uptake significantly and also ameliorated the decline in uptake of K^+. Increased uptake of mineral elements like N, K^+, Ca^{2+}, etc. impart beneficial effects on the plant growth by regulating photosynthesis, enzyme functioning, and tolerance mechanisms (Umar et al., 2011; Iqbal et al., 2015; Tahjib-Ul-Arif et al., 2018). Excess Na^+ accumulation reduces K^+ uptake hence reducing the K^+/Na^+ ratio (Umar et al., 2011) by directly affecting the expression of transport genes like *SOS*, *NHX*, etc. (Sun et al., 2015). Reduced uptake of Na^+ concomitant with increased K^+ uptake due to application of tocopherol may have contributed to increased growth, photosynthesis, and enzyme functioning, thereby leading to protection of metabolism under salinity; however, the exact mechanisms are still unknown. *Arabidopsis thaliana* plants exhibiting deficient tocopherol biosynthesis are sensitive to salinity and display restricted growth accompanied by excessive Na^+ accumulation and hence Na^+/K^+, oxidative damage and reduced PSII activity (Ellouzi et al., 2013).

Chlorophyll and Photosynthesis increases due to α-tocopherol application

In addition to reduced Na^+ uptake due to exogenous application of α-tocopherol increase in the content of photosynthetic pigments, total chlorophylls and carotenoids were evidently reflected in increased photosynthetic rate and PSII functioning. In corroboration to our finding Ali et al. (2019) have also demonstrated increase in chlorophylls, carotenoids and photosynthetic rate in wheat under drought stress. Enhanced photosynthesis and PSII functioning due to exogenous application of tocopherol in present study confirms the regulation of stomatal and non-stomatal attributes of photosynthetic machinery (Ali et al., 2019). Reduced chlorophyll synthesis and photosynthesis due to salinity stress has been reported by others as well (Taibi et al., 2016; Iqbal et al., 2015). However, the ameliorative

effects of exogenous tocopherol have rarely been reported. Salinity drastically declines the chlorophyll synthesis by down-regulating the functioning of enzymes mediating chlorophyll synthesis (Tarun and Tripathy, 2015). Stresses decline the functioning of key enzymes like Rubisco, Rubisco-activase and reduces the assimilation and partitioning of N for synthesis of Rubisco hence inducing decline in photosynthesis (Iqbal et al., 2015; Mo et al., 2016). Reduced α-tocopherol is a strong lipid antioxidant that contributes to the protection of PSII against stress-induced photodamage (Spicher et al., 2017). In present study it was obvious that α-tocopherol at both concentrations improved photosynthesis with impact much obvious and significant at 500 µM. Moreover, increased antioxidant functioning due to applied tocopherol may have also contributed to photosynthetic protection by quick neutralization of various ROS (Fritsche et al., 2017).

α-Tocopherol application reduces oxidative damage by up-regulating antioxidant system

Improved photosynthetic performance in plants treated with tocopherol can be attributed to reduced oxidative damage in them. In the present study, it was observed that accumulation of ROS including H_2O_2 and $O_2^{\cdot-}$ declined significantly due to tocopherol application causing an enhancement in membrane stability. Besides this tocopherol-mediated amelioration of oxidative damage was obvious as declined lipoxygenase activity, which is significantly up-regulated under stressed conditions (Nahar et al., 2016). Increased accumulation of ROS triggers peroxidation of lipids, thereby causing leakage of essential cellular constituents, including ions (Nahar et al., 2016; Elkelish et al., 2019a, b), however, tocopherol mediated declined lipid peroxidation and increased membrane stability confirms its beneficial effect on improving the stability of proteins and lipids thereby protecting the structural and functional integrity of membranes (Ali et al., 2019). Reduction in ROS (H_2O_2 and $O_2^{\cdot-}$) accumulation in root tissue of soybean due to tocopherol supplementation has been reported by Serefliogu et al., (2017). The singlet oxygen scavenging in chloroplast by α-tocopherol results in the generation of key compounds like α-tocopherol quinone, which contributes to cyclic electron transport in the thylakoid membrane hence providing photoprotection to chloroplasts (Munne-Bosch and Alegre, 2010). Earlier salinity-induced enhancement in ROS accumulation, lipoxygenase, and lipid peroxidation has been reported by others as well (Nahar et al., 2016; Ahanger et al., 2019). Reduction in the oxidative effects triggered by salinity stress due to exogenous application of tocopherol can be attributed to the up-regulation of the antioxidant system (Orabi et al., 2017), which was also obvious in the present study. In the present study, it was observed that exogenous application of tocopherol up-regulated the activity of antioxidant enzymes and the content of non-enzymatic antioxidants. Salinity enhanced the activity of antioxidant enzymes like SOD, APX, and GR, and similar results have been reported earlier (Iqbal et al., 2015; Elkelish et al., 2019a). In the present study, the activities were further enhanced by the application of tocopherol, depicting the strengthening of the tolerance system for quick elimination of ROS. Neutralization of excess ROS is important for protecting the structural and functional integrity of key cellular macromolecules and hence the whole plant (Soliman et al., 2019). Differential activation of antioxidant enzymes in soybean due to tocopherol treatment under salt stress has been reported (Serefliogu et al., 2017). SOD is unique for the dismutation of superoxide radicals and hence provide protection to photosynthetic machinery by maintaining ROS levels for better signaling communication between chloroplast and nucleus (Foyer, 2018). However, the elimination of excess H_2O_2 is mediated by APX and GR in an intriguing pathway that involves the active participation of GSH and AsA. It was interesting to observe that exogenous application of tocopherol up-regulated the components of AsA-GSH pathway, thereby mediating quick elimination of ROS particularly H_2O_2, within chloroplast and mitochondria. Earlier, Orabi and Abdelhamid, (2016) have also reported up-regulation of the antioxidant system by applying α-tocopherol in *Vicia faba* treated with saline water. In wheat, significant amelioration of oxidative damage triggered by water deficit due to up-regulation of enzymatic and non-enzymatic components of the antioxidant system by foliar application of α-tocopherol was observed (Ali et al., 2019). The availability of AsA and GSH influences the functioning of APX and GR in addition to perform scavenging of ROS and maintain redox homeostasis for protection of key functions like electron transport (Sakhno et al., 2019). Maintaining optimal functioning of AsA-GSH maintains NADP/NADPH ratio and the functioning of PSII and PSI (Pandey et al., 2015; Ahanger et al., 2017).

α-tocopherol application maintains the osmoregulatory components and secondary metabilte synthesis

Besides, exogenous application of α-tocopherol resulted in increased accumulation of sugars, proline, and GB under normal conditions. Salinity induced accumulation of sugars, proline and GB was further enhanced by the application of α-tocopherol. Earlier, increased accumulation of sugars and proline due to salinity stress has been reported by Ahanger and Agarwal (2017) and Elkelish et al. (2019a) in wheat plants. Increased accumulation of GB due to salinity stress has been reported by Khan et al. (2014), resulting in increased growth and photosynthetic performance. Under salinity stress tocopherol treatment reduced proline accumulation in soybean however triggered accumulation under normal condition (Serefliogu et al., 2017). Such results are contradictory to present study specifically under salinity stress conditions. Increase synthesis of tocopherol in transgenic alfalfa plants imparts stress tolerance by enhacing the accumulation of osmolytes inlcuding proline and sugar (Ma et al., 2020). Enhancement in the accumulation of osmolytes directly results due to modulated metabolism wherein their biosynthesis is increased manifold over their catabolism (Elkelish et al., 2019a). In the present study, it was observed that the activity of γ-GK increased due to the application of exogenous α-tocopherol reflecting in the greater synthesis of proline. In saline stressed *Vicia faba*, Orabi and Abdelhamid, (2016) had reported increased proline accumulation due to exogenous application of α-tocopherol resulting in significant amelioration of damaging effects on growth. Similar results of the increased γ-GK activity causing enhanced proline accumulation has been reported earlier (Iqbal et al., 2015). An increase in the sugar content of *Calendula officinalis* due to foliar application of tocopherol has been reported by Soltani et al., (2012); however, influence under salinity has not been reported. Compatible osmolytes mediate maintenance of water relations, enzyme functioning, and stress signaling, thereby protecting the major cellular processes, including photosynthesis, by eliciting quick stress response (Hayat et al., 2012; Liang et al., 2013; Annunziata et al., 2017; Den Ende, 2019; Qin et al., 2021). Osmolytes interplay with key molecules and their structures, and protect the activities of macromolecules, and maintain the integrity of membranes by scavenging ROS (Mansour and Ali, 2017). Compatible osmolytes strengthen the antioxidant system and have an immense contribution to ROS elimination when enzymatic antioxidants weaken. Exogenously applied tocopherol mediated enhancement in osmolyte content can be used as a potential strategy for averting the salinity-induced damage. In addition to this, the content of total phenols and flavonoids was enhanced due to the application of tocopherol; however impact of salinity stress was non-significant. Earlier, increased accumulation of phenols and flavonoids due to exogenous application of tocopherol has been reported in drought-stressed wheat (Ali et al., 2019) and maize (Ali et al., 2020) plants.

Secondary metabolites like phenols and flavonoids have a key role in plant protection under stresses and contribute to antioxidant property considerably (Ahanger and Agarwal, 2017; Krol et al., 2014). Phenolic compounds have an important role in plant development, especially in lignin and pigment biosynthesis, and provide structural integrity and scaffolding to plants (Bhattacharya et al., 2010). Increased accumulation of phenols and flavonoids prevents stress-induced oxidative effects by assisting in eliminating ROS, thereby protecting the structural and functional integrity of key macromolecules (Sharma et al., 2019). Besides, secondary metabolites have a strong antioxidative property (Khalid et al., 2019), which was also evident from the DPPH radical scavenging functioning in the present study.

α-tocopherol strengthens glyoxylase system

The damaging effects of salinity stress-triggered oxidative stress were further facilitated by upregulated functioning of glyoxylase system in tocopherol treated seedlings. Glyoxylase system eliminates the toxic MG hence preventing its toxic effects on cellular functioning (Mahmud et al., 2020). The upregulated activity of glyoxylase enzymes has been reported by Nahar et al. (2016) and Soliman et al. (2019) under salinity and Ni stress, respectively. Increased activity of glyoxylase enzymes causes a significant decline in the accumulation of MG, thereby protecting the structural integrity of cells. Strengthening of glyoxylase system due to exogenous application of Zn and Ca (Ahmad et al., 2018) and β-aminobutyric acid (Mahmud et al., 2020) has been reported to enhance the salinity tolerance in *Brassica juncea* and *Brassica napus* plants, respectively. MG is a mutagenic and cytotoxic compound known to initiate growth arrest and also reacts with proteins, DNA and increases sister chromatid exchange (Sankaranaraanan et al., 2017). Therefore tocopherol mediated up-regulation of glyoxylase system lessens the accumulation of MG and hence the damaging effect on growth.

Conclusion

Conclusively, exogenous application of α-tocopherol at higher concentrations proved effective in mitigating the damaging effects of salinity stress in soybean plants. Declined accumulation of ROS and Na^+ accumulation in α-tocopherol treated plants significantly reduced the oxidative effects on membrane functioning and photosynthesis. The up-regulated antioxidant functioning, glyoxylase system, and osmolyte accumulation contributed significantly to improved salinity tolerance in the tested plant. The beneficial effects of α-tocopherol triggered under normal conditions were maintained even under salinity stress conditions thereby providing enough strength to withstand the stress.

Conflicts of interest

No potential conflict of interest was reported by the authors.

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