APPLICATION OF TRIACONTANOL TO INCREASE SALINITY TOLERANCE IN SUNFLOWER (*HELIANTHUS ANNUUS* L.)

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Abstract

Triacontanol is a natural plant growth regulator and increases the physiological efficiency of plants under stress conditions. A pot experiment was conducted to investigate the role of triacontanol on growth of sunflower under saline conditions. Two sunflower cultivars Hysun-33 (V1) and S-278 (V2)) were sown in pots containing a well-mixed soil. After ten days of seed germination, salinity levels (0, 60, 120 mM) were created with NaCl according to the saturation percentage of the soil. After two weeks of salinity treatment, different levels of triacontanol (0, 20, 40 µM) were applied as a foliar spray along with tween 20 as surfactant. Saline growth medium reduced the plant growth, chlorophyll and protein contents and increased the accumulation of amino acid and total soluble sugars. Results showed that effectivity of triacontanol in reducing the salinity effect on physiology and biochemistry of treated sunflower plants.

Key words: Protein, Triacontanol, Salinity, Sunflower

Introduction

Salinity is considered as major environmental threats for agriculture (Rahman *et al*., 2017; Islam *et al*., 2020). There are two main threats to agriculture sustainability i.e., increasing human population and reduction in land availability for cultivation (Shahbaz and Ashraf, 2013). Worldwide, approximately 930 million hectares of arable land, representing more than 6% of total land area, are salt-affected and this percentage is increasing daily mainly by natural and anthropogenic activities (Hasanuzzaman *et al*., 2018; Islam *et al*., 2020). The loss of land due to salinity will be up to 50 % by 2050 (Jamil *et al*., 2011; Roy *et al*., 2012). More than 800 Mha land is salt affected all over the world (Munns and Tester, 2008). In Pakistan, 4.8 Mha of irrigated land is affected by salinity stress (Ashraf, 2010). Soil salinity is one of the worlds wide problem that limit plant growth and productivity (Canama *et al*., 2013; Kumar *et al*., 2017; Klein *et al*., 2018). Under salinity stress, soils reduces the absorption of water by the plant. As result, the water potential of the plant is lowered (Koyro *et al*., 2011). Ionic toxicity occurs when a toxic amount of Na^+ and Cl^- accumulate in the leaves. A high amount of sodium and chloride in the root zone affects the vital processes of the plant such as water uptake, photosynthesis, enzyme activities, ion accumulation, hormonal balances, cell division and expansion and disorganize the membrane structure by producing toxic chemicals (Deivanai *et al*., 2011; Namjooyan *et al*.,2012; Klein *et al*., 2018; Islam *et al*., 2020). Reactive oxygen mostly produced under high saline conditions that cause oxidative stress in plants. Oxidative stress damages lipids, proteins, nucleic acid and ultimately whole machinery of the cell (Namjooyan *et al*., 2012; Chen *et al*., 2017). Plants adopt different strategies for stress tolerance. Production of antioxidants is one of the best strategies to combat with reactive oxygen species (Gill and Tuteja, 2010). Many researchers have reported exogenous application of growth substances to alleviate the adverse effects of salt stress (Khan *et al*., 2009; Iqbal *et al*., 2011;; Habib *et al*., 2016; Ahmad *et al*., 2019). Antioxidants detoxify or scavenge the reactive oxygen species and protect the plant from oxidative stress. Triacontanol (TRIA) is one of the most effective antioxidants to protect the plant from stress (Shahbaz *et al*., 2013; Islam *et al*., 2020).

Triacontanol plays an important role in enhancing growth, photosynthesis, yield of plants, water and nutrients uptake, nitrogen fixation, enzyme activities, and increasing the concentration of free amino acids reducing sugar and soluble protein organic compounds under stress conditions (Singh *et al*., 2011; Perveen *et al*., 2013; Khandaker *et al*., 2013; Li *et al*., 2016; Naeem *et al*., 2017; Sharma *et al*., 2018). In earlier studies foliar applied triacontanol has shown reduction in the adverse effects of various abiotic stresses e.g., Basil (Borowski *et al*., 2000; Shahbaz *et al*., 2013; Zulfiqar and Shahbaz, 2013; Perveen *et al*., 2014, 2017) and canola (Zulfiar and Shahbaz 2013; Maresca *et al*., 2017) and improved membrane integrity by differentially modulating membrane lipid composition (Islam *et al*., 2020). Many researchers have reported that exogenous application of TRIA inhibits lipid peroxidation in

spinach and peanut plants (Ramanarayan *et al*., 2000; Khan *et al*., 2009). Exogenous application of TRIA-improved the growth and also provide tolerance by boosting the activity of antioxidant enzymes like POD under salt stress (Perveen *et al*., 2017; Islam *et al*., 2020).

Sunflower (*Helianthus annuus* L.) is an important oil-seed crop all over the world. Globally, 13 % of the total edible oil is produced from sunflower and is moderately sensitive to soil salinity. (Forleo *et al*., 2018; Lalarukh and Shahbaz, 2020). In Pakistan, the sunflower is cultivated on 376.11x103ha area (GOP., 2009). Present research work was conducted to determine the role of triacontanol in increasing salinity tolerance of sunflower cultivars Hysun-33(V_1) and S-278(V_2).

Materials and Methods

The under study experiment was conducted on sunflower (*Helianthus annuus* L.) in the Department of Botany, University of Sargodha, Pakistan. The experiment was conducted to assess the effect of exogenous application of Triacontanol on growth, physiological and biochemical aspects of sunflower grown under saline and non-saline conditions. Salinity treatments (0 mM, 60 mM, 120 mM) were created with NaCl based on soil saturation percentage. Foliar spray of Triacontanol was applied after 2-weeks of salt treatment.

Estimation of chlorophyll content: Chlorophyll contents were determined after fifteen days of triacontanol application by using the method of Arnon (1949) and Davies (1976). Fresh leaves of (0.5 g) were chopped into segments of 0.5 cm and extracted with 5 ml acetone (80 %) at 10 ^{0}C overnight. Material was centrifuge at 14000 x *g* for 5 minutes and measured the absorbance of the supernatant at 645, 652 and 663 nm on spectrophotometer. Chlorophyll a, chlorophyll b, total chlorophyll and carotenoid were calculated by using formulas.

Chlorophyll a (mg/g. f. wt.) = [12.7(ΔA 663)-2.69(ΔA 645)] × V/1000 ×W

Chlorophyll b (mg/g. f. wt.) = [22.9(ΔA 645)-4.68 (ΔA 663)] ×V/1000 × W

Total Chlorophyll (mg/g. f. wt.) = [20.2(ΔA 645) +8.02 (ΔA 663)] ×V/1000 × W

Carotenoid (mg/g. f. wt.) = ΔA 480 + (0.114 × ΔA 663) - (0.638 × ΔA 645) ×V/1000 × W

ΔA = Absorbance at respective wavelength

V = Volume of extract (ml)

W = Weight of sample (g)

Total soluble protein: Total soluble proteins were determined using the method of Lowry *et al*. (1951). 1mL of the leaf extract from each treatment was taken in a test tube. The blank contained 1mL of phosphate buffer (pH 7.0). 1mL of solution C was filled in each test tube. The reagents in the test tube were gently mixed. After that test tubes were kept at room temperature for 10 minutes. Then 0.5mL of Folin-Phenol reagent (1:1 diluted) was added in each test tube, mixed well and incubated for 30 min. at room temperature. The optical density (OD) was read at 620 nm on a spectrophotometer (UV-1700, PharmaSpec, Shimadzu Japan).

Total free amino acids: Total free amino acids were determined according to Hamilton and Van Slyke (1973). Plant fresh leaves (0.5 g) were chopped and extracted with phosphate buffer (0.2 M) having pH 7.0. One mL of the extract was taken in 25 mL test tube, added one mL of pyridine (10 %) and 1mL of ninhydrin (2 %) solution in each test tube. Ninhydrin solution was prepared by dissolving 2 g ninhydrine in 100 mL distilled water. The test tubes containing sample were heated in boiling water bath for about 30 min. Volume of each test tube was made up to 50mL with distilled water. Read the optical density of the coloured solution at 570 nm using spectrophotometer. Developed a standard curve with Leucine and calculated free amino acids using the formulae given below:

$$\text{Total amino acids } (\mu g\ g^{-1}\ f.\ wt) = \frac{\text{Graph reading of sample x Volume of sample x Dilution factor}}{\text{Weight of fresh tissue x 1000}}$$

Nitrate reductase activity (NRA): Nitrate reductase activity was determined according to Sym (1984). In 10mL of 0.02 M phosphate buffer, 0.5 g of leaf samples was added. One mL of medium was taken and 1 mL of 0.02 M KNO_3 was added and incubated in dark for 30 min. at 30 ^{0}C. After incubation, 0.5 mL of 1 % sulphanilamide and immediately after shaking 0.5 mL of 0.02 % (1-naphthyl) ethylene diamine dihydrochloride were added. Pink diazo complex was produced with NO_2. Then the color was noted and diluted the samples if necessary. Absorbance was measured by spectrophotometer at 542 nm.

Determination of Protein: The proline was estimated by using the Bates *et al*. (1973) method. Toluene was used as a blank. Absorbance was read at 520 nm using spectrophotometer.

Total soluble sugars: Yemm and Willis (1954) method was used to determine the total soluble sugars. Extract of plant was put in 25 mL test tubes. 6 mL of anthrone- reagent was added in each tube. Test tubes were heated for 10 minutes in boiling water bath. The test tubes were allowed to be ice-cooled for 10 min. and again incubated for 20 min. at room temperature

(25ºC). Optical density was read at 625 nm on the spectrophotometer (UV-1700, PharmaSpec, Shimadzu Japan). The concentration of soluble sugars was calculated from the standard curve.

Statistical analysis: Analysis of variance of the data from each attribute was computed using the MSTAT Computer Program. The Duncan's New Multiple Range test at 5 % level of probability was used to test the differences among mean values (Steel and Torrie, 1980).

Results

Analysis of variance of data for chl. a presented in (Table. 1). Salinity stress markedly reduced the chl.a of both sunflower cultivars Hysun-33 (V1) and S-278(V2). Foliar spray of Triacontanol mitigated the adverse effects of salt stress. Among different levels of TRIA, 40µM was more effective to increase the chl. a concentration of both sunflower cultivars (Fig. 1).

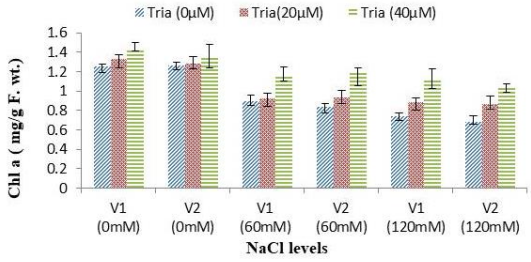
Chlorophyll b, total chl and carotenoid concentrations were also reduced under the saline conditions (Fig. 2, 3 and 4). However, foliar application of Triacontanol significantly increased the concentration of chl. b, total chlorophyll and carotenoids (Fig. 2, 3 and 4). 40µM level of TRIA was more effective to increase all these attributes. Total soluble sugar was increased under saline conditions as has been indicated in fig.5.

Analysis of variance of the data for total free amino acid, total soluble protein, proline, nitrate reductase activity, achene per plant and weight of achene were presented in (Table. 2). Saline growth medium decreased the total proteins, number of achene and achene weight of both sunflower cultivars (Fig.7, 9, 10 and 11) and increased the free amino acid, proline, (Fig.8). Exogenous application of Triacontanol significantly improved the total soluble proteins of both sunflower cultivars and yield of the plants. Salinity stress decreased the activity of nitrate reductase enzyme. Foliar application of Triacontanol enhanced the activity of NRA. Contents of total free amino acids were increased under saline growth medium. Foliar spray of Triacontanol was not effective to increase the total free amino acids of both sunflower cultivars. Total soluble sugars and proline concentration was increased under saline growth medium. Triacontanol effectively increased the total soluble sugars and proline of both sunflower cultivars (Table. 2, Fig. 2). Triacontanol enhanced the number and weight of achenes of sunflower (Fig. 10 and 11). Overall, Hysun-33 was more salt tolerant as compared to S-278.

Table.1: Analysis of variance (ANOVA) of data of chl a, chl b, total chl and carotenoid concentrations to determine the effect of TRIA on sunflower cultivars under saline and non-saline conditions

Source of variations	DF	Chl. a	Chl. b	Total chl	carotenoids	Total soluble sugar
Variety	1	0.0234*	0.0202*	0.093^{**}	1.794*	0.394^{*}
Treatment	2	0.875**	0.114**	1.716**	0.002**	35.682^{**}
V X T	2	0.004^{NS}	0.0002^{NS}	0.0008^{NS}	3.506^{NS}	0.038^{NS}
Error	48	0.028	0.006	0.0376	4.488	0.075
Total	53					

Note:* Significant at P≤0.05; ** Significant at P≤0.01; NS non-significant



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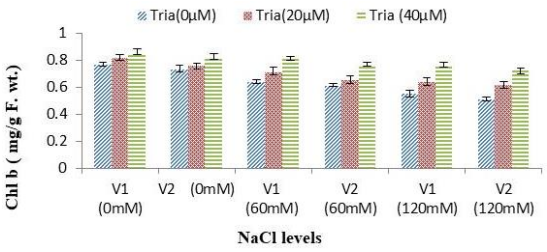

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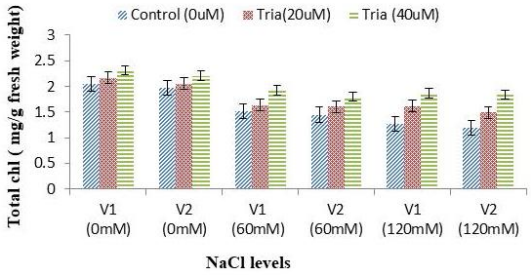

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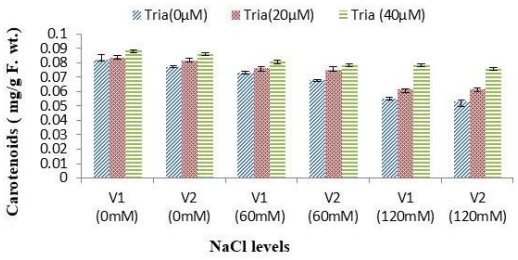

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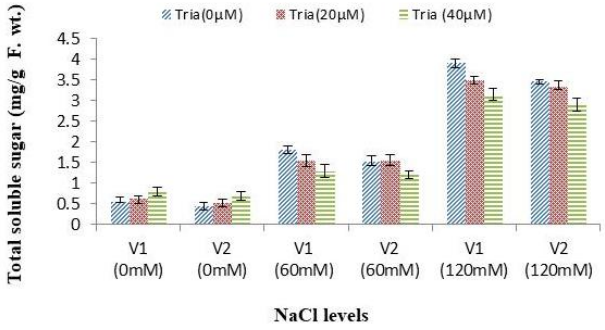

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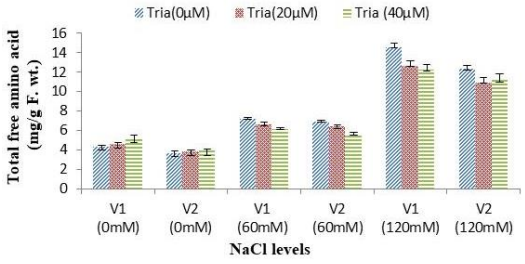
Fig.1, 2, 3, 4 and 5: Effect of Triacontanol on chlorophyll a, chlorophyll b, total chlorophyll and carotenoids and total soluble sugar concentration in sunflower (*Helianthus annuus* L.) under saline and non-saline conditions

Table. 2: Analysis of variance (ANOVA) of data of total protein, nitrate reductase activity, total free amino acids, total soluble sugars and proline concentrations to determine the effect of TRIA on sunflower cultivars under saline and non-saline conditions

Source	DF	Total free amino acids	Total soluble protein	Proline	NRA	No. of achenes/plant	Wt. of achenes/plant
Variety	1	11.986**	1.775*	0.558**	0.702**	1745.4*	3.303*
Treatment	2	318.606**	23.587**	14.837**	6.787**	38079.2**	55.155**
V X T	2	1.849^{NS}	0.096^{NS}	0.049^{NS}	0.042*	17.4^{NS}	0.409^{NS}
Error	48	0.581	0.401	0.0226	0.079	424.3	0.576
Total	53						

Note:* Significant at P≤0.05; ** Significant at P≤0.01; NS non-significant



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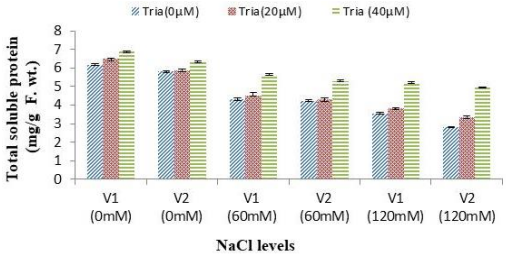

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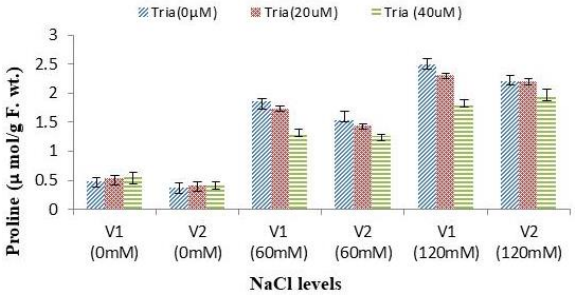

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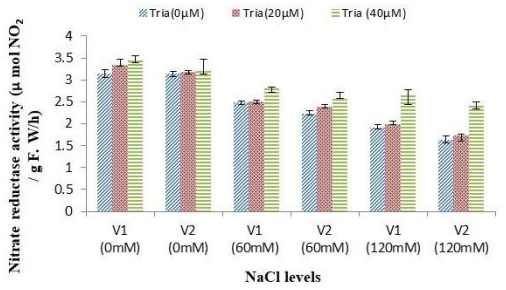

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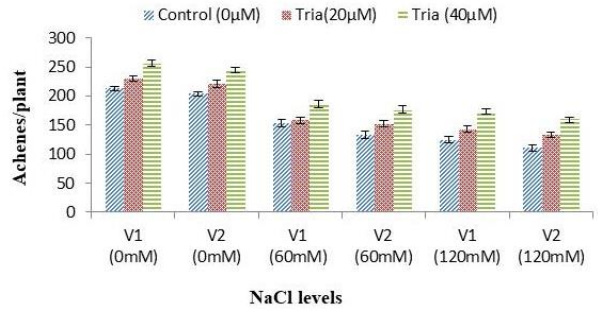
Fig. 6, 7, 8 & 9: Effect of Triacontanol on total soluble sugar, total free amino acids, total protein, nitrate reductase activityand proline of sunflower (*Helianthus annuus* L.) under saline and non-saline conditions



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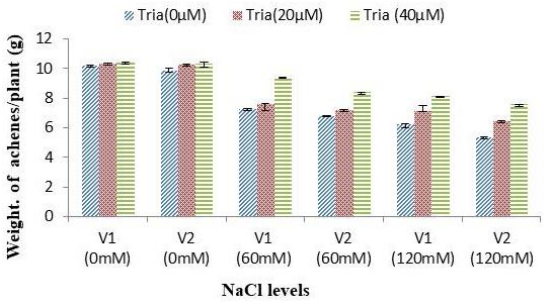

(11)

Fig. 10 and 11: Effect of Triacontanol on total free amino acid, total soluble proteins, proline, nitrate reductase activity, achene and achene weight on sunflower (*Helianthus annuus* L.) under saline and non-saline conditions.

Discussion

Soil salinity is a great challenge to agricultural crops at global level (Munns *et al*., 2010). The enhancement in crop growth and yield is necessary to feed the population, growing at a rapid rate. Salinity stress adversely changes the physiological, biochemical and molecular attributes of plant, thus resulting in reduced crop productivity (Takahashi *et al*., 2009; Chen *et al*., 2017).

Generally, high salt concentration in growth medium causes hyperosmotic and hyperionic effects and harshly reduce the plant growth (Turan, 2010). Salinity stress decreased the plant growth by reducing the plant biomass, plant height; chlorophyll contents (Akram *et al*., 2010). Under study experiment showed

that salinity stress decreased the growth of sunflower. This reduction may be due to osmotic stress (Shabala *et al*., 2012), ionic toxicity (Shahbaz and Zia., 2011) and hormonal imbalance (Babu *et al*., 2012; Ahmad *et al*., 2019).

Exogenous application of osmoprotectants, plant growth regulators, antioxidants, organic and inorganic compounds has been found very effective to mitigate the adverse effects of salinity stress on different crops (Liu and Shi., 2010). Triacontanol (TRIA) effectively ameliorates the harmful effects of salt stress and regulates the plant growth under saline and non-saline conditions (Naeem *et al*., 2011). Under study experiment showed that exogenous application of Triacontanol (20µM & 40µM) as foliar spray increased growth, as measured root fresh and dry weight and shoot fresh and dry weight in both sunflower cultivars (Hysun-33 & S-278).

The growth enhancement in various crops by the application of Triacontanol has been investigated by many scientists, i.e. tomato and ginger (Khan *et al*., 2009; Singh *et al*., 2011). During under study experiment, Triacontanol showed improvement in growth may be due to up-regulation of genes that are related to photosynthetic process (Singh *et al*., 2011), and improvement in antioxidant enzyme activities (Perveen *et al*., 2012, 2014).

Salinity stress suppresses the photosynthetic rate. Decrease in photosynthetic process may be due to stomatal closure (Tavakkoli *et al*., 2010), and inhibition of photochemical capacity and reduction in CO_2 assimilation reported by (Hussain *et al*., 2008). Under study experiment resulted that foliar spray of Triacontanol increased the Chl a, Chl b, total Chl and carotenoid concentration in both sunflower cultivars under saline and non-saline conditions.

High concentration of sodium in plant tissues competes with the uptake of other essential nutrients such as potassium (Talei *et al*., 2012). High uptake of K^+ as compared to Na^+ might be considered as salinity tolerance (Dhingra, 2014). Accumulation of Ca^{2+}, Mg^{2+}, P and K^+ reduces under salt stress. Under study experiment investigated that exogenous application of Triacontanol increased the uptake of Ca^{2+}, Mg^{2+}, K^+ and P under saline and non-saline condition by reducing the uptake of Na^+ and Cl^-. Same results have been reported by (Krishnan and Kumari., 2008). Under salt stress, osmoprotectants such as proline and soluble sugar accumulate to protect the plant. osmoprotectants increase tolerance against salinity stress. It is basic strategy adopted by plants to protect themselves against salt stress (Sabir *et al*., 2011). In previous studies, high accumulation of proline has been observed in salinity stressed sunflower plants (Bajehbaj, 2010). In under study experiment accumulation of proline, total free amino acids and soluble sugar was substantially increased under saline growth medium. Exogenous application of Triacontanol also enhanced the accumulation of osmoprotectants.

Excessive amount of salt in soil adversely affects the enzymes activity and regulate the nitrogen metabolism. The reduction in nitrate reductase activity (NRA) and nitrite reductase activity (NiRA) is attributed to reduced rate of enzyme synthesis and direct inhibition (Iqbal *et al*., 2006). In current study, results revealed that salt stress decreased the activity of nitrate and nitrite reductase enzymes while the foliar application of Triacontanol enhanced the activity of these enzymes under salt stressed environment.

Improvement in crop productivity is major issue all over the world. Salinity stress has been reported to decrease yield of major crops such as wheat (Shahbaz *et al*., 2008). Under study experiment showed that sunflower yield was decreased under salinity stress. Foliar application of TRIA increased the sunflower yield under saline and non-saline conditions.

In conclusion, salinity stress exerted the negative effect on growth, photosynthesis, biomass production, essential nutrient accumulation and yield of both sunflower cultivars. Biochemical attributes like activity of nitrite reductase enzyme, nitrate reductase enzyme and protein contents decreased under saline environment. Salinity stress enhanced the accumulation of total free amino acids, total soluble sugars and proline of both sunflower cultivars.

However, foliar application of TRIA significantly enhanced the plant height, plant fresh and dry biomass, chlorophyll contents, essential ion accumulation, osmoprotectant accumulation and yield of both sunflower cultivars. Among different Triacontanol levels, 40 µM was found to be more effective to enhance the growth and yield attributes of sunflower cultivars. Overall, Hysun-33 was more salt tolerant than S-278 and showed better performance in response to Triacontanol.

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