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RESEARCH ARTICLE

INVESTIGATIONS INTO THE EFFECT OF SALICYLIC ACID ON SOME BIOCHEMICAL AND ANTIOXIDANT PARAMETERS IN SOYBEAN UNDER SALT STRESS

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ABSTRACT

Salinity is one of the major abiotic stresses which are responsible for limiting crop production. Foliar spray with hormones has been an effective method for increasing growth and productivity of plants grown under salt stress. Salicylic acid regulates many physiological processes in plants when subjected to environmental stresses. Soybean cultivar Pusa- 9712 plants were grown with 150 mM NaCl were sprayed with 10^{-4} M, 10^{-5} M, or 10^{-6} M SA and some physiological processes were studied to authenticate our understanding of their role in tolerance to salinity-induced oxidative stress and also to see how much these parameters are induced by SA application. Results depicted that salt stress negatively affected photosynthetic pigments and protein content and enhanced lipid peroxidation in both control and stressed plants. Foliar application of SA increased the Superoxide dismutase (SOD), Guaiacol peroxidase (POD) and Catalase (CAT) activity in salt stressed plants. CAT activity was found to be highest in plants treated with 10^{-6} M as compared to control whereas same concentration of SA increase proline content by 89.04% to mitigate the impact of salt stress. Result further exhibited that lipid peroxidation was also reduced by about 21.53% under salt treatment. Infact, SA treatment prevents the negative effects of salt stressed soybean and this could be adopted as a potential growth regulator to improve soybean grown under salt stress.

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INTRODUCTION

Soil affected with salinity conditions has been reported from different climatic zones and at different heights above sea level (Rao *et al*. 2006). Salinity, among abiotic stress, is one of the main factors that limit plant growth and development in both arid and semi-arid regions. Salt stress negatively effects the plants growth and development by decline in chloroplast activity, inhibiting photosynthetic rate and increase photorespiration rate which then leads to production of increased reactive oxygen species (ROS) as reported by Parida and Das (2005). Soybean is an important protein crop of the world due to its high protein (35%), edible oil (21%) and carbohydrate (35%) and its production may be limited by various abiotic stresses such as soil salinity (Ghassemi-Golezani *et al*., 2009). Plants have different mechanism to reduce the effect of stress. Foliar application of compounds is one way to reduce negative effects of abiotic stresses (Yuan *et al*., 2008).

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During stress occurrence, salicylic acid is a messenger molecule which plays a non- enzymatic antioxidant role in regulating plant physiological mechanisms (Arfan *et al*., 2007). Also, SA is known for its ameliorative effects in inducing salt tolerance in many crop plants (Tayeb 2005, Stevens 2006). Foliar spray of SA stimulates the growth and development has been reported in wheat (Shakirova *et al*., 2003), soybean (Gutierrez-Coronado *et al*., 1998) and maize (Gunes *et al*., 2007). Samina *et al.,* (2009) also reported that oxidative stress was reduced by applying SA exogenously at saline condition. Hence, an experiment was conducted to study whether foliar application of SA could ameliorate salt stress in soybean by modulating various biochemical parameters and antioxidative enzymes.

MATERIAL AND METHODS

Plant material and growth conditions

Seeds of Soybean variety (Pusa- 9712) were obtained from CCS Haryana Agriculture University, Hissar. The experiment was set up in the experimental cage of Department of Botany,

Kurukshetra University, Kurukshetra. Five seeds per pot were surface sterilized and sown in earthen pots (30 cm diameter) lined with polythene having 5.0 kg of dune sand grown under natural light conditions during kharif season in 2014 and 2015. The temperature conditions were 35 ± 2°C and 24 ± 2°C, during days and nights respectively; with mean relative humidity of 82 ± 5%.

Treatments

After three weeks, the seedlings were thinned to two plants per pot and each treatment consisted of three replications in a complete randomized design (CRD). Fifteen days before taking the sample foliage of the plants was sprayed uniformly either with double distilled water (control), or with different concentrations (10^{-4}M, 10^{-5} M, 10^{-6} M) of SA dissolved in ethanol to elucidate the effect of exogenous SA on plants. The plants were sampled at 75 DAS to assess various biochemical parameters.

Estimation of chlorophylls and carotenoids

Leaf sample (200 mg) was ground in chilled 80% acetone (AR grade) with 20 mg of $CaCO_3$ and centrifuged at 3000 g for 5 min. Absorbance of the filtrate was recorded at 645 and 663 nm for chlorophylls and at 480 and 510 nm for carotenoids depending upon respective peaks in their absorption spectra using a UV-Visible spectrophotometer (Specord-205, Analytic-Jena, Germany). Chlorophyll (Chl) amount was estimated with the formula of Arnon (1949). Carotenoid level was calculated by the method of Holden (1965).

Estimation of total soluble protein

Total soluble proteins were estimated according to the method described by Bradford (1976) using Coomassie Brilliant Blue G-250. Fifty mg of fresh leaf tissue (earlier stored in a freezer) was dropped boiling 80% ethanol (EtOH) on a water bath for a minute. The tissue along with EtOH was cooled to room temperature and homogenized. The extract was centrifuged at 10,000 g for 5 min. The residue was re-extracted with 5% perchloric acid followed by centrifugation at 10,000 g for 5 min. Five-mL of 1N NaOH was added to the residue and maintained in warm water (40-50°C) with regular shaking for 30 min. The clear supernatant was used for further analysis.

Measurement of peroxidase (POD) activity

Total peroxidase activity was measured by the method of Maehly (1954). Plant material (0.1 g) was homogenized with ice cold distilled water and centrifuged in a Remi centrifuge at 6000 g for 10 min. The supernatant was used as the enzyme source and final volume of the extract raised to 10 mL with ice cold double distilled water. The reaction set was prepared by mixing 2 mL each of enzyme source; phosphate buffer (pH 7.0); guaiacol (20 mM), and H_2O_2 (10 mM) in sequence. A blank set was prepared by mixing 2 mL of enzyme source; 2 mL of phosphate buffer (pH 7.0) and 4 mL of double distilled water. Blank, and reaction sets, were kept undisturbed at room temperature exactly for 10 min., then the absorbance was recorded in a spectrophotometer at 420 nm. Protein was estimated from the same extract following the procedure of Bradford (1976).

Measurement of superoxide dismutase (SOD) activity

Fifty-mg of fresh leaf tissue was crushed in 2 mL of 0.1M EDTA- phosphate buffer, pH 7.8, containing K_2HPO_4 and EDTA and the final volume raised to 100 mL with double distilled water (DDW). This was centrifuged at 15000 g and the resultant supernatant used as crude extract. The reaction mixture was prepared by adding 0.1 mL of crude extract followed by 0.9 mL of DDW, 0.5 mL of 300 mM Na_2CO_3 (pH 10.2), 0.5 mL of 378 µM p-nitrobluetetrazolium chloride (NBT), 0.5 mL of 78 mM L-methionine and 0.5 mL of 7.8 µM riboflavin. The final reaction mixture was 3 mL. The reaction was carried out in test tubes at 25°C for 15 min under 100 µmol photon $m^{-2}s^{-1}$ PF from fluorescent lamps. The initial rate of reaction, measured by the difference in increase in absorbance at 560 nm in the presence, and absence, of extract was proportional to the amount of enzyme. The unit of SOD activity was obtained as that amount of enzyme which under the experimental conditions caused a 50% inhibition of the reaction observed in the absence of enzyme (Giannopolitis and Ries, 1977).

Measurement of Lipid peroxidation

The level of lipid peroxidation in samples was measured by estimating the malondialdehyde (MDA) present (Heath and Packer, 1968). Leaf samples (0.2 g) were homogenized in 3 mL of 50 mM phosphate buffer (pH 7.0). The homogenate was centrifuged at 15000 g for 15 min. To 1.0 mL aliquot of the supernatant, 2.0 mL of 0.5 % thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) was added. The mixture was heated at 95°C for 30 min in a water bath and then cooled in an ice bath. After centrifugation at 10000 g (Remi) for 10 min the absorbance of the supernatant was recorded at 532 nm. The value for nonspecific absorption of each sample at 600 nm was recorded and subtracted from the absorbance recorded at 532 nm.

Catalase (CAT) activity

The supernatant that was used for APX activity was also used to determine catalase activity (CAT) following the method of Aebi (1984). The reaction mixture was prepared by adding 1.5 ml of 50 mM HEPAS buffer 1.2 ml of 150 Mm H_2O_2 and 30 µl petal extract. In the reaction mixture without enzyme, no crude extract was added, instead of it 50 µl 50 mM HEPAS buffer was added. The change in absorbance was read at 490 nm in the test tube cuvette using uv- vis spectrophotometer. Specfic activity of catalase was expressed in term of per mg protein. Protein was estimated from the same extract following the procedure of Bradford (1976) as described earlier.

Proline content

The proline content was determined using the method described by Bates *et al*., (1973). Proline was extracted from leaf sample of 100 mg FW with 2 ml of 40% methanol, 1 ml of the extract was mixed with 1ml of a mixtue of glacial acetic

acid and rothophosphoric acid (6 M) ((3: 2, v/v) and 25 mg of ninhydrin. After 1 h incubation at 100°C, the reaction was terminated by putting the tubes in ice bath, 5 mL toluene was added. The absorbance of the upper phase was spectrophotometrically determined at 520 nm. The proline concentration was determined using a standard curve.

Statistical analysis

A mean of three readings was taken in every replication. In biochemical estimation, three aliquots were used for each replication. Statistical analysis was done using Statistical Packages for Social Sciences (SPSS) version 16.0. One -way ANOVA was used to test whether there was a significant difference in various estimations.

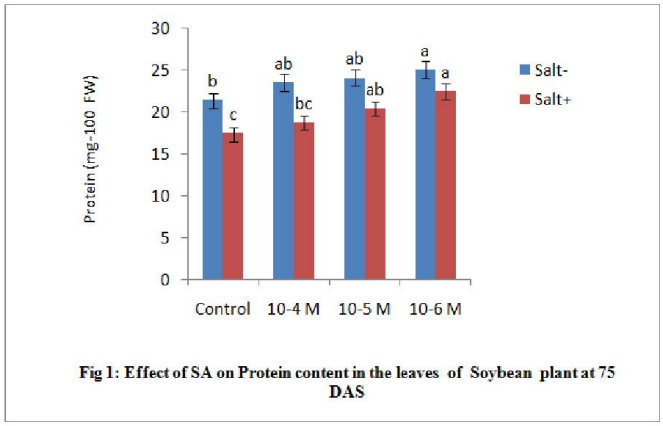

Fig 1: Effect of SA on Protein content in the leaves of Soybean plant at 75 DAS

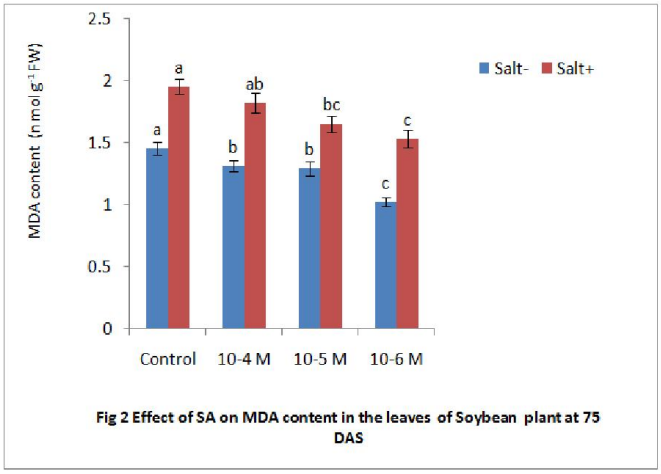

Fig 2 Effect of SA on MDA content in the leaves of Soybean plant at 75 DAS

RESULTS AND DISCUSSION

Photosynthetic pigments: Salinity stress significantly declined the photosynthetic pigments (Chl. a, Chl. b, Total Chl. and carotenoids) by 24.40 %, 27.63 %, 28.34 %, and 37.19 % respectively in comparison to control of non-treated

Table 1. Effect of different concentration of salicylic acid (10^{-4}, 10^{-5} and 10^{-6} M) on the amount of Chl.a, Chl.b, total Chl. and Carotenoids (mg/g fresh wt.) in Soybean at 75 DAS

Salinity level	Stages of sampling	Treatments	Chlorophyll a	Chlorophyll b	Total chlorophyll	carotenoids
		Control	$3.36^{d}\pm 0.11$	$1.52^{d}\pm 0.10$	$5.08^{e}\pm 0.17$	$2.42^{bc}\pm 0.08$
		SA(10^{-4} M)	$3.92^{c}\pm .0.15$	$1.68^{c}\pm 0.08$	$5.60^{e}\pm 0.23$	$2.56^{abc}\pm$ 0.09
(0 mM)	75	SA(10^{-5} M)	$4.34^{b}\pm 0.17$	$1.85^{b}\pm 0.07$	$6.19^{b}\pm 0.25$	$2.72^{ab}\pm 0.07$
		SA(10^{-6} M)	$4.54^{a}\pm 0.12$	$1.90^{a}\pm 0.09$	$6.95^{a}\pm 0.23$	$2.92^{a}\pm 0.09$
		Control	$2.54^{e}\pm 0.06$	$1.10^{e}\pm 0.05$	$3.64^{f}\pm 0.12$	$1.52^{f}\pm 0.04$
		SA(10^{-4} M)	$2.89^{e}\pm 0.10$	$1.23^{d}\pm 0.10$	$4.12^{ef}\pm 0.16$	$1.82^{ef}\pm 0.06$
(150 mM)	75	SA(10^{-5} M)	$3.34^{d}\pm 0.11$	1.38d± 0.08	$4.76^{d}\pm 0.16$	$1.95^{def}\pm 0.05$
		SA(10^{-6} M)	$3.75^{c}\pm$ 0.12	$1.53^{c}\pm 0.07$	$5.28^{c}\pm 0.18$	$2.10^{cde}\pm 0.06$

Data are means ± SE of three replicate. Means followed by the same letter for each tested parameter are not significantly different (P< 0.05)

plants at 75 DAS after sowing as shown in (Table- 1). However, foliar application with SA enhanced the accumulation of chlorophyll in both stressed and non-stressed plants. Under non-stressed condition, SA increased the Chl. a by 35.12 %, Chl. b by 25.01% , Total Chl. by 36.21 % and carotenoids by 20.66 % as compared to control at 10^{-6} M whereas salinized plants when sprayed with SA increased the Chl. a by 47.63 %, Chl. b by 39.09 %, Total Chl. by 45.13 % and carotenoids by 38.15 % as compared to control at 10^{-6} M. Foliar application of SA at 10^{-5} M also retained the photosynthetic pigments but to the lesser extent than 10^{-6} M. Interestingly SA at 10^{-4} M was negligible in retaining the pigment loss.

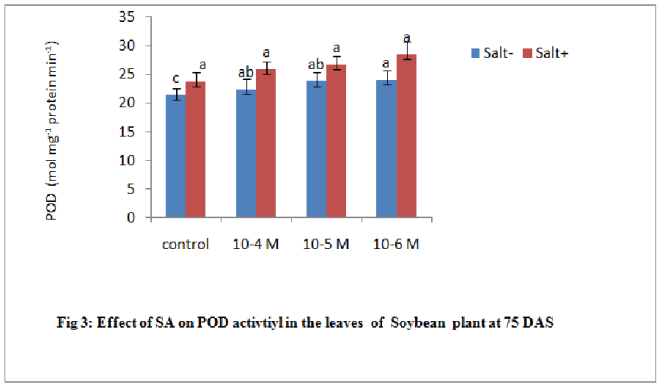

Fig 3: Effect of SA on POD activtiyl in the leaves of Soybean plant at 75 DAS

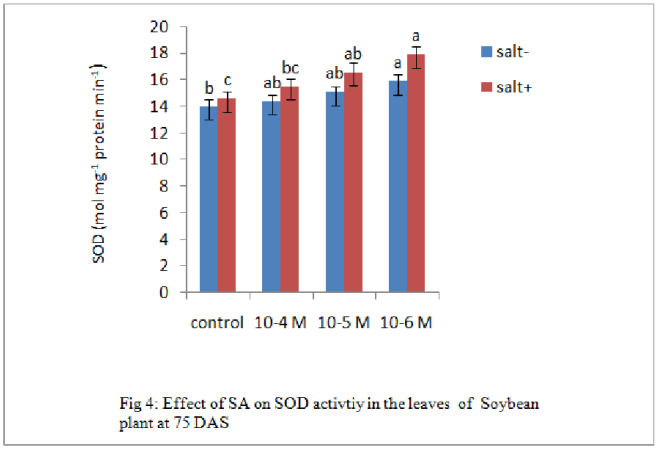

Fig 4: Effect of SA on SOD activtiy in the leaves of Soybean plant at 75 DAS

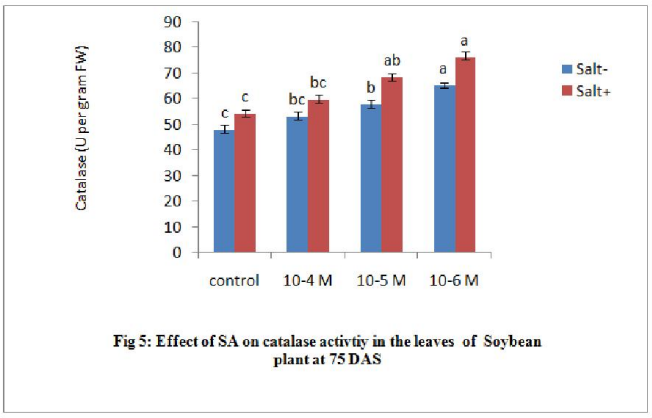

Fig 5: Effect of SA on catalase activtiy in the leaves of Soybean plant at 75 DAS

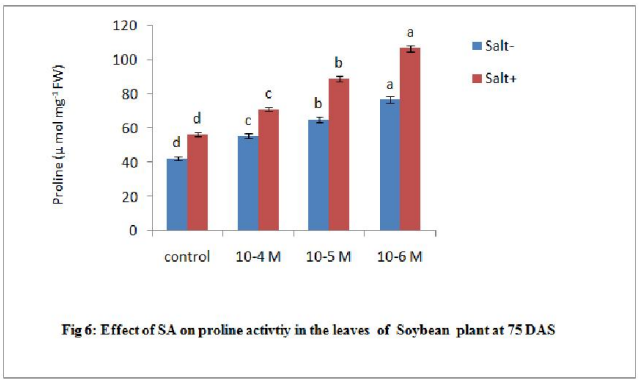

Fig 6: Effect of SA on proline activtiy in the leaves of Soybean plant at 75 DAS

The stimulatory effects of SA on photosynthetic pigments in our study are in agreement with those obtained by Barakat *et al*., (2011) on wheat ; Saeidnjad *et al*., (2012) on maize. In another study on *Brassica juncea*, (Fariduddin *et al*., 2011) reported that net photosynthetic rate enhanced by foliar spraying of salicylic acid as well as intracellular CO_2, water use efficiency, stomatal conductance along with transpiration rate whereas high SA concentrations (1–5 mM) leads to reduction in the photosynthetic rate. Thus, at high doses of SA diminished the photosynthetic activity is due to its efficacy on the thylakoid membranes and light-induced reactions linked to them. In mustard seedling, a lower concentration of SA (10µM) also improves the photosynthetic net CO_2 assimilation. Also, salinity is known to fluster a multitude of physiological processes including photosynthesis. Such salt-induced reduction in photosynthesis is in agreement with the earlier findings in Brassica spp. (Nazir *et al*., 2001) and in wheat (Raza *et al*., 2006)

Protein content – In the present study, salinity stress decreased protein content of soybean plant but exogenous application of SA maintained this attribute not only in salt stress but also under optimum conditions. From the data of the present investigation (Fig. 1), it is clear that salinity stress decreased protein content in the leaves of soybean as compared to control of non-stressed plant by 18.63 %. However, foliar application of SA somewhat retained the protein content under both saline and non-saline condition. Treatment of SA at 10^{-6} M retained the protein content by 16.78 % in non-stressed plant whereas in stressed plant protein content was found to be increased by 28.69 % as compared to control. Foliar application of SA with 10^{-5} M or 10^{-4} M was not significant in retaining the protein content when compared to 10^{-6} M. In cowpea plant total soluble sugar and soluble protein found to be increased after application of SA as reported by (Chandra *et al.* 2007). Our observations are similar to Kumar *et al.* (1999) who reported that total protein content was found to be enhanced in soybean plants when sprayed with SA and this increase might be due to enhanced in the activity of nitrate reductase by following the SA application. Further, in another study, wheat leaves when treated with SA provided a significant protection to the enzyme nitrate reductase thereby keeping the normal level of proteins (Singh and Usha, 2003).

MDA content – As salt level increase, lipid peroxidation increase depends on different plant species (EI-Beltagi *et al*., 2008; Sadak *et al*., 2010). This enhancement in lipid peroxidation may be due to the fact that salinity could modify the membrane structure and stimulate o_2 production as mentioned by (Zhang *et al.,* 1996). Delvari *et al.,* (2010) reported that pretreatment of SA will decrease the level of lipid peroxidation generated by oxidative stress in basil plants. However, SA treatment of wheat leaves under water stress condition resulted in less production of MDA (Agarwal *et al.,* 2005). Foliar spraying of SA ameliorated the negative effects of abiotic stress in wheat (Shakirova *et al*., 2003), cucumber (Yildirim *et al.,* 2008) and for maize (Khodary, 2004) for maize. Salt stress caused membrane injury in the leaves of soybean seedlings, so under stress condition MDA content increased by 34.98 % when compared with that of control of non-stressed plants in (Fig. 2). However, SA prevented lipid peroxidation by alleviating the membrane injury of soybean seedlings. So, foliar application of SA reduced the lipid peroxidation by 29.65 % in non-stressed plant as compared to control whereas under salt- stressed condition, MDA content was inhibited by about 21.53 % as compared to control at 10^{-6} M. However, foliar spray with 10^{-4} M or 10^{-5} M was not significant in reducing the lipid peroxidation.

Antioxidant activities – In the present study, activities of SOD, POD and CAT in soybean seedlings were significantly affected by salt stress. Treatments with SA significantly enhanced the activities of SOD, CAT and POD under both stressed and non-stressed plants (Fig. 3-5). SA at 10^{-6} M significantly increased the activities of POD, SOD and CAT activities by about 12.44 % , 13.70 % and 36.60 % respectively at 75 DAS in comparison to control in non-stressed plant. However, under stressed condition, application of SA significantly enhanced POD, SOD and CAT activities by 20.58 %, 22.87 % and 41.06 % respectively as compared to control. In comparison of 10^{-6} M, SA at 10^{-4} M or 10^{-5} M did not generate positive effect on antioxidant activities except CAT. At 10^{-5} M SA, CAT activity was found to be increased by 26.02 % in stressed plant whereas by 20.73 % in non- stressed plant in comparison to control. These antioxidant enzymes and metabolites are reported to enhance under various environmental stresses (Sakr *et al.,* 2013) and this increment was also studied in the current work. Salinity stress levels clearly affected enzyme activity in pepper shoots. In plants at various concentration of SA like 10^{-7}M or 10^{-4}M antioxidant activities like guaicol peroxidase (POD), ascorbate peroxidase (APX), Superoxide dismutase (SOD) and glutathione reductase (GR) have all been reported to showing different changes (Sakhabutdinova *et al.,* 2003). Senaratna *et al.,* (2000) reported

that SA confers tolerance to pepper plants and the tolerance was associated with changes in antioxidants system. Sawada *et al.,* (2006) reported that salicylic acid (SA) is an important signal molecule against plant defense which is involved in regulation of the anti-oxidative system. The impact of abiotic stress found to be ameliorated by SA treatment by enhancing antioxidant system which is necessary to inhibit etiolating factors like oxidative damage and ion leakage from membrane (Yusuf *et al.,* 2008).

Proline content- Against salinity stress proline is one of the important components of the adaptation of plants (Abbaspour, 2012) and pretreatment with SA also contributed to accumulation of this amino acid under stress possibly through maintaining an enhanced level of ABA in the plants (Ervin, 2005). Salt stressed dramatically induced the accumulation of proline in the leaves of soybean plant. Treatment of SA further enhanced the proline content. Under stressed condition, application of SA at 10^{-6} M increased the proline content by 89.04 % whereas under non-stressed plant treatment of SA enhanced the proline content by 80.56 % as compared to control represent in (Fig. 6). Application of 10^{-5} M SA was also found to be effective whereby increasing the proline content by 57.58 % in stressed plant whereas in non-stressed plant proline content found to be enhanced by 53.03 % as compared to control. However, foliar application of SA at 10^{-4} M was non-significant in increasing the proline content.

Conclusion

In conclusion, our results showed that although common soybean is a moderately sensitive plant against salinity stress, however foliar application of SA (especially at 10^{-6} M) can help to increase the tolerance of this crop by maintaining chlorophyll and protein levels and also modulating antioxidant enzymes under salt stress.

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