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Physiological Tools to Identify the Amelioration Effect of Salicylic Acid Under Salinity in Sorghum at Bloom Stage

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Abstract

Little is known about the impact of salicylic acid in sorghum under salt stress. Sorghum genotype HJ 513 was used for the experiment, which was carried out as factorial in a completely randomized design. Plants were grown in screen houses under four salt levels (0, 5.0, 7.5 and 10.0 dS m-1 NaCl) and three salicylic acid, SA (0, 25 and 50 ppm) levels with twelve different combinations. Sorghum leaves were harvested at bloom stage (80 DAS) and assayed for electrolytic leakage percentage (ELP) i.e. 93.81% in HJ 513, osmolytes content (Proline value increased from 59.75 to 86.56 (µg g^{-1} DW) in HJ 513 under 10 dS m-1 with respect to control, total soluble carbohydrate content also increased from control to 10 dS m-1 i.e. 0.21 to 0.42 (mg g^{-1} DW) in HJ 513, glycine betaine content increased from control to 10 dS m-1 i.e. 135.67 to 286.63 (µmole g^{-1} DW) in HJ 513 The specific activities of the superoxide dismutase, catalase and peroxidase under salt stress (10 dS m^{-1}) increased 69.12%, 255.29% and 92.65% in HJ 513 respectively. Compared with the plants treated with salt alone, added salicylic acid significantly decreased ELP and significantly enhanced osmolytes concentration and also the antioxidant enzymatic activity in salt-stressed leaves of that genotype. That SA effect was time-dependent and became stronger as the experiment continued. It could be concluded that higher activities of SOD, CAT and POX in salt-stressed leaves induced by SA addition may protect the plant tissues from membrane oxidative damage under salt stress, thus mitigating salt toxicity and improving the growth of sorghum plants. The results of the present experiment coincided with the conclusion that SA may be involved in metabolic or physiological changes in plants.

Key words : Salicylic acid, Bloom stage, Sorghum, Antioxidant enzymes.

The largest growers of sorghum are India, America and Nigeria. India contributes 9.45% of the world's sorghum production with 5.82 million hectares area and 5.39 million tonnes of total production (Gite *et al*., 2015). The major constraints that reduce sorghum productivity are abiotic and biotic stress. Sorghum is moderately salt tolerant, that is well adapted in arid and semi-arid regions where salinity is the major problem. The reason for selecting sorghum for this study is: being a dual crop grown for both food and fodder. Salt stress toxicity is a worldwide agricultural and eco-environmental problem. Approximately one-third of the world land surface is arid and semi-arid, out of which one half is affected by salinity (Liang *et al*. 1996). In India, approximately 6.7 lakh hectare of the cultivated land area are adversely affected by salinity. In Haryana alone, it is 5 lakh hectare. Salt stress in plants retards their growth and results in a reduction of natural vegetation due to osmotic and ionic effects in soil solution. Short-term effects include a reduction on growth by salt due to osmotic effects. Long-term effects include excessive salt absorption, which causes plants to suffer ionic stress, leading to premature leaf aging following a reduction in the available photosynthetic area to maintain growth which was due to decreased activity of photosynthetic enzymes in Calvin cycle (Misra *et al*., 1997). The major constraints for plant growth and productivity are ion toxicity with excessive uptake of mainly Na^{+} as well as nutrient imbalance caused by disturbed uptake of

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essential mineral nutrients such as K^+. As a result of maintaining the cytoplasmic K^+ and Mg^{2+} concentrations at levels required for proper essential enzyme activities, these plants can achieve osmotic adjustment through the synthesis of osmoprotectants and prevent the buildup of toxic levels of reactive oxygen species (ROS) which generally occurs under saline conditions. In many salt-tolerant species the increased contents of amino acids such as proline and glycine betaine were observed. malondialdehyde (MDA) accumulates rapidly (Fadzilla *et al*. 1997, Hernandez *et al*. 1993, Lutts *et al*. 1996), which increases the permeability of plasma membranes, so which leads to reducing the water potential of soil solution and decreases plant ability for water uptake. Furthermore, the inevitable excessive absorption and accumulation of salts in plants experiencing salinity cause ion imbalance and toxicity (Ashraf and Harris, 2004); (Parida and Das, 2005). Salt stress in plants is also associated with many secondary physiological changes such as reduction of enzyme activity, an increase of oxidative stress, membrane dysfunction, reduced photosynthesis and other morpho-anatomical alterations (Parida and Das, 2005); (Tester and Davenport, 2003). Adverse environmental conditions cannot be tolerated by plants and they are not able to move from one place to another, so for survival, plants do some metabolic changes under stress conditions like a synthesis of some organic metabolites or some growth regulators. According to Iqbal and Ashraf (2007), stress tolerance is achieved by the application of these growth regulators in plants. A new scheme for amelioration salt stress is to overcome the irregularities in plant physiological mechanisms by some of the plant growth regulators and nutrients. According to Senaratna *et al*. (2000), plant growth and development and their interaction with other organisms is controlled by salicylic acid (SA).

Salicylic acid is classified as a phenolic compound a plant hormone endogenous regulator, which can regulate plant growth and also provides protection against biotic and abiotic stresses such as salt stress (Kaya *et al*., 2002). The effect of salicylic acid on plant physiological processes varies and depends on species, developmental stage and environmental conditions (Shraiy and Hegazi, 2009). Salicylic acid application influences a wide variety of plant processes, including stomatal regulation, chlorophyll content, photosynthesis (Yildirim *et al*., 2008) and also seed yield (Khodary, 2004). The ameliorative effect of SA on the growth of crop plants under abiotic stress conditions may have been due to its role in nutrient uptake (Glass 1974), water relations (Barkosky and Einhelling 1993), stomatal regulation (Larque-saavedra, 1979; Arfan *et al*., 2007), photosynthesis and growth (Khan *et al*., 2003; Arfan *et al*., 2007). Evidence indicates that SA is an endogenous signal molecule for the activation of plant growth and plant defense responses to systemic acquired resistance and pathogen attack local (hypersensitive response; Klessig and Malamy, 1994). It has been shown that SA can markedly improve germination under salt stress, and exogenously applied SA can significantly increase plant growth under both saline and non-saline conditions, particularly at the 500 uM/L level (Kováˇcik *et al*., 2009). Therefore, an experiment was conducted to determine whether foliar-applied SA could induce salt tolerance in sorghum plants and to draw relationships between antioxidant enzyme activity and osmolytes concentration to elucidate the mechanism associated with improved salinity tolerance in sorghum due to exogenously applied SA. The objective of this research is to determine the physiological response (ELP, osmolytes content and the activities of the antioxidant enzymes) associated with enhanced tolerance resulting from the application of SA to salinity stress.

Material and methods

Plant Material, Treatment and Plant Growth Conditions : Seed of sorghum genotype (HJ 513) was collected from Forage Section, Department of Genetics and Plant Breeding, Chaudhary Charan Singh Haryana Agricultural University, Hisar (Haryana). Dune sand was taken from gangwa village. Each plastic pot was filled with 10 kg sand. Then the seeds of sorghum were sterilized in 1% sodium hypochlorite (NaOCl) solution for 5 min to avoid contamination before using them for experimentation. Ten seeds were sown in each pot of 25 cm diameter with equal depth and distance containing 10 kg well-washed sand. After germination, seedlings were thinned to three of equal size. According to Hoagland and Arnon, (1950), hoagland nutrient solution was used to each pot after every week and moisture content of the sand was maintained daily by adding 200 ml distilled water to each pot. Before sowing, pots were saturated with desired levels of salt i.e. Control (0), 5.0, 7.5 and 10.0 dS m-1. The control pots were irrigated with canal water. Salicylic acid (0, 25 and 50 mg l-1) will be applied exogenously with the help of manual sprayer after 70 days after sowing (DAS). Present research work was carried out in Department of Botany and Plant physiology, CCS HAU, Hisar; to mitigate the adverse effects of salt on electrolyte leakage, osmolytes concentration, and activity of antioxidant enzymes in sorghum genotype by the foliar application of salicylic acid. Arrangement of the experiment was completely randomized design with three replicates. The sampling was done at vegetative stage.

Membrane permeability (Electrolyte leakage) : This parameter was included to have more information on the membrane stability and thereby on the relative ion content in the apoplastic space. Electrolyte leakage was assessed as described by Sullivan and Ross (1979). Leaf samples were collected from control as well as treated plants. One hundered mg of leaf tissue was taken separately in 20 ml test tube containing 10 ml of de-ionized water. These samples were incubated for 3-4 hr at room temperature. The conductance of decanted liquid containing effluxed electrolytes was determined with a conductivity meter and designated as ECa (Before boiling). Then the samples were subjected to heating at 1000C in a water bath for 30 minutes. After cooling, the electrical conductivity of the solutions was measured and designated as ECb (After boiling). The electrolyte leakage was expressed by the following formula:

$$\text{Electrolyte leakage (\%)} = \frac{EC_a}{EC_b} \times 100$$

Determination of compatible osmolytes content : The proline content of cell free extract was estimated by the method of Bates *et al*. (1973). Three hundred mg of leaf tissue was homogenized in 3ml of 3% aqueous sulpho-salicylic acid and the residue was removed by centrifugation at 12,000 rpm for 10 min then 2ml of the supernatant was taken then add 2 ml acid-ninhydrin and 2 ml of glacial acetic acid in a test tube. Test tubes were kept in water bath for 1 hour at 100°C and the reaction is terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene, mixed vigorously and left at room temperature for 30 min until separation of the two phases. The chromophore-containing toluene (upper phase) was warmed to room temperature and its optical density was measured at 520 nm using toluene as blank. The proline concentration was determined from a standard curve using L-Proline.

Total soluble carbohydrates were determined with the method of Yemm and Willis (1954), using anthrone reagent. An aliquot from the

extract, which was made by using 80 % ethanol, measuring 0.2 ml was evaporated to dryness in a test tube in a boiling water bath. On cooling the residue left in the tube was dissolved in 1 ml of distilled water and mixed with 4.0 ml of the anthrone reagent. The mixture was heated in a water bath for 10 minutes. After cooling, absorbance was recorded at 620 nm using Spectrophotometer. Standard curve was prepared using graded concentration (20-100 µg ml^{-1}) of D-Glucose and the data was expressed as mg g^{-1} DW.

Glycine betaine was estimated according to the method of Grieve and Grattan (1983). Leaf extract was prepared in 20 ml of test tubes by chopping 0.5 g leaves in 5 ml of 0.05% toluene. All the tubes were kept for 24 hr at 25°C. After filteration 0.5 ml of extract was mixed with 1 ml of 2 N HCl solution and 0.1 ml of potassium tri-iodide solution (containing 7.5 g iodine and 10 g potassium iodide in 100 ml of 1 N HCl) was added and shaken in ice cold water bath for 90 min and then 2 ml of ice cold water was added after gentle shaking and then 10 ml of 1, 2-dichloroethane (chilled at -10°C) was pour in it. By passing continuous stream of air for 1-2 minutes two layer were separated, upper aqueous layer was discarded and optical density of organic layer was recorded at 365 nm. Standard curve was prepared using graded concentration of glycine betaine and the data were expressed as µmole g^{-1} DW of the tissue.

Specific activity of antioxidant enzymes: Specific activity of SOD was estimated by the method of Giannopolitis and Ries (1977) with little modifications. The reaction mixture contained 1 ml of enzyme extract and to this added 0.5 ml of each of methionine, NBT, EDTA and Na_2CO_3 and the total volume of 4 ml was made with buffer in each set adjusting the pH at 10.2 then 0.5 ml of riboflavin was added to each set in the last. The tubes were shaken and placed 30 cm from light source (8 x 20 W fluorescent lamps). The reaction was allowed to run for 10 minutes and then stopped by switching off the light. The tubes were immediately covered with a black cloth. The absorbance was recorded at 560 nm and it is expressed as unit mg^{-1} protein min^{-1}. Catalase (CAT) activity was estimated by the method of Aebi (1983). Enzyme extract was made with 0.1M phosphate buffer (pH 7.0), five hundred µl of extract was taken and to this added 0.2 ml of 0.1 M H_2O_2 and 1.5 ml of 50 mM potassium phosphate buffer. The enzyme sample was added immediately at the time of taking the absorbance and incubated for 3 minutes. The change in absorbance was recorded at 240 nm at an interval of 15 seconds for 1.5 minute. The specific activity of enzyme was expressed as unit mg^{-1} protein min^{-1}. The procedure of Siegel and Siegel (1986) was followed for estimating peroxidase activity. Three ml of reaction mixture contained 0.1 M phosphate buffer (pH 7.0), 0.1 mM guaiacol and 100 µl cell free extract. Reaction was started with the addition of 0.1 mM H_2O_2 and increase in absorbance at 470 nm was recorded for 2 min. The activity was calculated using the extinction coefficient value of 26.6 mM^{-1} cm^{-1} for guaicol. One unit of enzyme activity was equivalent to µmol of H_2O_2 consumed per minute during the reaction.

Statistical analysis : The data were analysed statistically for ANOVA using complete randomized design (CRD) by using OPSTAT programme. Treatments were compared with CD values at 5% level of significance.

Results and discussion

Electrolyte leakage in two genotype of sorghum was higher at salt stress treatment as compared to unstressed condition. Several authors like Kukreja *et al*. (2006) and Rani (2004) reported that electrolyte leakage increased consistently with increasing levels of

salt stress due to the accumulation of H_2O_2 content. McNeil *et al*. (1999) observed that percent increase in electrolytes under salt stress was due to the displacement of Ca^{2+} membrane protein and also change in the composition of membrane lipids, which alter membrane permeability. Increment in electrolyte leakage by 93.81% in HJ 513, as compared to the control under salt stress (10.0 dS m^{-1}) in HJ 513, shown in Fig. 1. However, exogenous SA application (50 mg l^{-1}) reduces the leakage of electrolytes as compared to the control under salt stress (10 dS m^{-1}) i.e., by 19.93% in HJ 513. Foliar application of SA decreased the electrolyte leakage under salt stress as well as in control condition. Clarke *et al*. (2004) stated that SA caused an overall decline in electrolyte leakage explaining its role in the maintenance of membrane integrity by electrostatic binding with negatively charged phospholipid head.

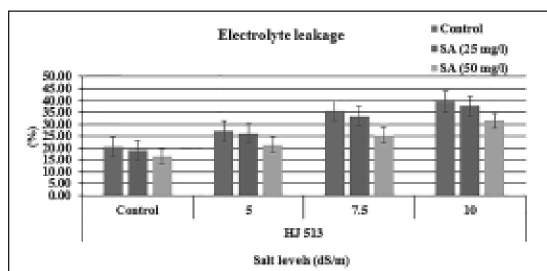

Fig. 1. Effect of salt stress on Electrolyte leakage (%) and its mitigation by salicylic acid in sorghum genotype HJ 513 at 80 DAS

Figure 2, 3 and 4, shows the effect of salt stress and SA treatments on total soluble carbohydrate, proline and glycine betaine content in sorghum genotype. The concentration of proline content was affected by salinity and SA treatments ($p < 0.05$). Salt stress increased the proline and glycine betaine content as compared to the non-saline conditions. Increased proline content showed positive relationship with decreasing water potential of leaf and it is also increased with the possible way through increased proteolysis. Increased proline accumulation was also noticed by the various workers i.e. Durgaprasad *et al*. (1996), Ashraf (1997), Singh (2003), Kukreja *et al*. (2006) and Nandwal *et al*. (2000, 2007).

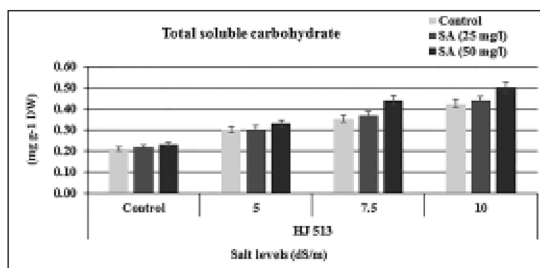

Fig. 2. Effect of salt stress on total soluble carbohydrate (mg g^{-1} DW) and its mitigation by salicylic acid in sorghum genotype HJ 513 at 80 DAS

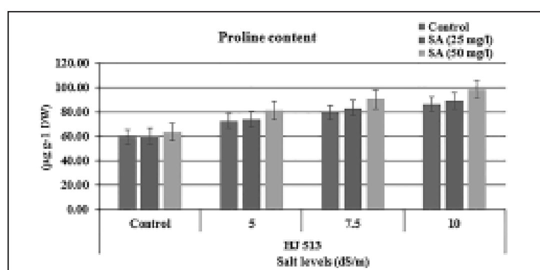

Fig. 3. Effect of salt stress on prline content (µg g^{-1} DW) and its mitigation by salicylic acid in sorghum genotype HJ 513 at 80 DAS

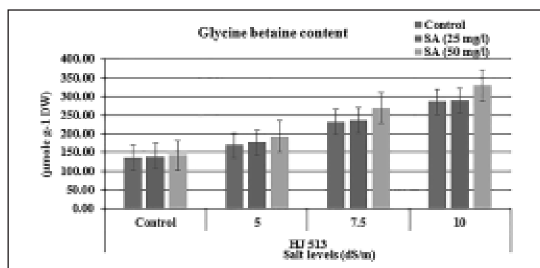

Fig. 4. Effect of salt stress on glycine betaine content (µmole g^{-1} DW) and its mitigation by salicylic acid in sorghum genotype HJ 513 at 80 DAS

Inze and Montagu (1995), also reported the accumulation of proline for osmotic adjustment which is an adaptive mechanism under salt stress. Sakhabodinova *et al*. (2004) and Arfan (2007) also reported proline increase in wheat under saline condition. Glycine betaine has main role to protect the membrane, enzymes and also to stabilize photosystem II protein pigment complexes under stressful conditions (Papageorgiou and Morata, 1995). The glycine betaine content also increased under stress condition in barley (Nakamura, 2001) and in Radix astragali (Tan *et al*. 2006). Total soluble carbohydrate was significantly increased with the increasing levels of salt stress from control to 10 dS m^{-1} sorghum at 80 DAS. This increase was from control to 10 dS m^{-1} i.e. 0.21 to 0.42 in HJ 513 (Fig. 2). This result was also favoured by Singh (2003), Kukreja *et al*. (2006) and Nandwal *et al*. (2007), suggested that increased level of total soluble carbohydrates is due to the breakdown of starch. Application of 50 mg l^{-1} SA also caused increase in total soluble carbohydrates in sorghum under stressed as well as in un-stressed conditions in HJ 513 genotype. Similarly, increase in the total soluble sugar was observed after exogenous application of SA to drought stressed shallot plants (Ahmad *et al*., 2014). Kaur *et al*. (2011) studied in Indian mustard that total soluble carbohydrates increased after foliar application of salicylic acid. Increase in proline content was estimated in stressed as well as in un-stressed conditions after application of SA. Maximum increment was noticed at 50 mg l^{-1} of salicylic acid in sorghum i.e. value increase from 86.56 to 98.43 in HJ 513 at 10 dS m^{-1} of salt level (Fig. 3). Proline is one of the important compounds of defense reactions of plants under salt stress, it must be expected that pretreatment with salicylic acid contributes to this amino acid accumulation under stress through maintaining an enhanced level of ABA in seedlings (Kuznetsov and Shevyakova, 1999). However, glycine betaine values also increased after the foliar application of both concentration of salicylic acid under stressed as well as in un-stressed plants. But at 50 mg l^{-1} of SA brought more increment in glycine betaine value i.e. 286.63 to 329.30 in HJ 513 at 10 dS m^{-1} of salt level (Fig. 4). Foliar application of salicylic acid also increased all the osmolytes under salt stress. Accumulated glycine betaine might serve as an intercellular osmotic balance and it can be closely correlated with the elevation of osmotic pressure (Kavikishore *et al*., 1995).

Figure 5, 6 and 7 showed that antioxidant enzyme activity dramatically increased due to salt stress. The lowest and highest activity was observed in control and 10 dS m^{-1} salt stress treatments, respectively. The activity of SOD significantly increased in stressed plants

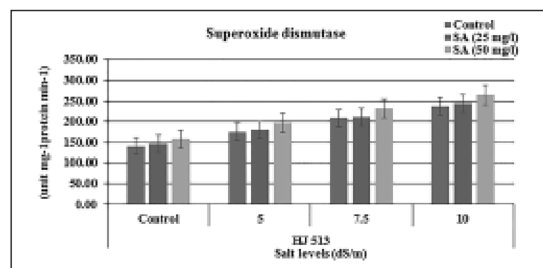

Fig. 5. Effect of salt stress on superoxide dismutase (unit mg^{-1} protein min^{-1}) and its mitigation by salicylic acid in sorghum genotype HJ 513 at 80 DAS

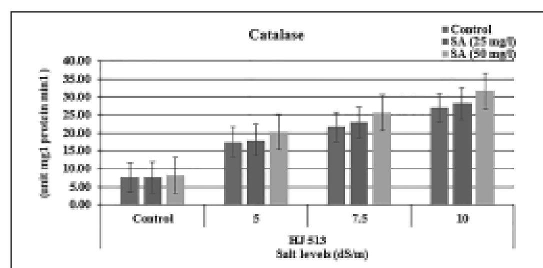

Fig. 6. Effect of salt stress on catalase (unit mg^{-1} protein min^{-1}) and its mitigation by salicylic acid in sorghum genotype HJ 513 at 80 DAS

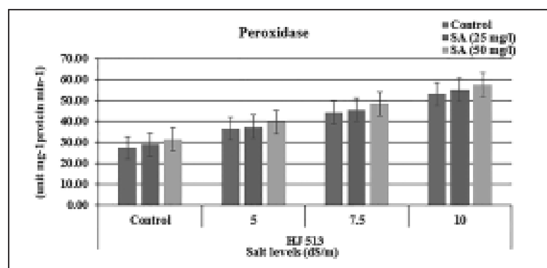

Fig. 7. Effect of salt stress on peroxidase (unit mg^{-1} protein min^{-1}) and its mitigation by salicylic acid in sorghum genotype HJ 513 at 80 DAS

compared to control plants in sorghum. Maximum per cent increase was noticed at 10 dS m^{-1} of salt level like 69.12% in HJ 513 with respect to control (Fig. 5). Similar results have been obtained by Maksimova and Matukhin (1965) on stressed millet leaves. Specific activity of catalase (unit mg^{-1} protein min^{-1}) increased with every increment of salt level (from control to 10 dS m^{-1}) in sorghum at 80 DAS. Per cent increase in specific activity of catalase was calculated in HJ 513 (255.29%) at 10 dS m^{-1} of salt level (Fig. 6). Catalase is tetrameric heme containing enzyme that catalyze the reaction by converting hydrogen peroxide into water and oxygen. The present results are same with earlier findings in wheat (Sairam *et al*., 2002) and chickpea (Singh *et al*., 2001 and Kukreja *et al*., 2006). Per cent increase in peroxidase activity was observed in HJ 513 (92.65 %) at 10 dS m^{-1} of salt level with respect to control (Fig. 7). Similar increase in activity of peroxidase was seen under salt stress in chickpea by Kukreja *et al*. (2006). Many researchers i.e. Meloni and Martínez (2009), Chernane *et al*. (2015) and Mickky and Aldesuquy (2017) have also been advocated the increase in specific activity of all the enzymes (SOD, CAT and POX) under salt stress. Application of salicylic acid further enhanced the specific activity of SOD, CAT and POX under salt stress which is clearly seen in our research findings. Foliar spray of SA (25 and 50 mg l^{-1}) brought significant increment in the specific activity of SOD in sorghum, but more increment was observed at 50 mg l^{-1} in HJ 513 (12.38%) with respect to their control at 5 dS m^{-1} of salt level (Fig. 5). Exogenous application of SA of both concentrations, significantly enhanced the specific activity of catalase under stressed and un-stressed conditions, but this increase was more towards 50 mg l^{-1} of SA under stressed condition (Fig. 6). Treatment with SA enhanced the specific activity of POX, but more enhancement was noticed at 50 mg l-1 of SA in stressed as well as in un-stressed conditions and this increase was on higher side at 7.5 dS m^{-1} of salt level in sorghum (Fig. 7). The specific activity of all the enzymes increased after application of SA because synthesized ROS must be mitigated. Similar observations were also noticed by Ebrahimian and Bybordi (2012). SA is a fundamental requirement for successful mitigation of salt stress because of ROS produced under salt stress damage the membrane structure, protein and DNA structure, which ultimately leads to death. Similar results were observed by He *et al*. (2005) in Kentucky blue grass. Based on these findings, the SA treatments may ameliorate the negative effect of salinity on the growth of sorghum. The addition of SA could offer an economical and simple application to the salt sensitive plant of sorghum production problems in aridisol caused by high salinity but further studies are required in order to determine the efficiency of these materials under natural field conditions.

Conclusion

Taking the above parameters into consideration it can be concluded that salt stress reduced the morpho-physiological and biochemical parameters leading to ultimately reduction in productivity in sorghum (HJ 513). The reduction was calculated based upon higher electrolyte leakage, lesser accumulation of osmolytes and increased antioxidative activites

with increasing levels of salt stress from control to 10 dS m^{-1}. Deleterious effects were more pronounced at higher level of salt (10 dS m^{-1}) stress. Better ameliorating effect was seen at 50 mg l^{-1} of SA based on the lower values of electrolyte leakage (%)and higher accumulation of osmolytes and further increment in antioxidative activites. Hence, this study concluded that the sorghum genotype showed better response in mitigating salt stress with foliar application of 50 mg l^{-1} SA.

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