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# EFFECT OF SALICYLIC ACID IN SOYBEAN (*GLYCINE MAX* L. MERIL) UNDER SALINITY STRESS

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## ABSTRACT
The present investigation was carried out to elucidate the mechanism of salinity tolerance by exogenous supply of salicylic acid at two concentrations viz. 100 and 200 ppm under created salinity stress of 50 mM and 100 mM NaCl in the soil. To check the efficacy of salicylic acid under salinity at 45, 60 and 75 days after sowing, foliar spray was done to study on biochemical parameters which lead to significant increase in chlorophyll content as well as sugar and starch content at the level of 100 ppm salicylic acid. Proline, one of the stress induced amino acid at concentration of 200 ppm also showed increased level indicating its potential role in combating salinity stress. Similar results were obtained for antioxidative enzymes like SOD and CAT at the level of salicylic acid at 200 ppm.Thus, it can be concluded that application of salicylic acid at varying concentrations of 100 and 200 ppm can lead to overcome salinity situations upto a certain extent.

## INTRODUCTION

Among various crops tested, legumes have generally been found to be more sensitive to salinity. Soybean is an important grain legume due to its high protein (35%), oil content (21%) and nitrogen fixing ability (17-24 kg/ha/yr.) and this grain legume is also highly sensitive to salinity. Wide genetic variability exists among different cultivars of soybean (Shereen and Ansari 2001). Nevertheless, soybean production may be limited by environmental stresses such as soil salinity (Ghassemi-Golezani *et al.,* 2009). The plants under saline condition change their metabolism to overcome the salinity stress. One mechanism utilized by the plants for overcoming the salt stress effects might be via accumulation of compatible osmolytes, such as proline and soluble sugar. Production and accumulation of free amino acids, especially proline by plant tissue during drought, salt and water stress is an adaptive response. Several lines of evidence demonstrate the alleviating role of salicylic acid during salinity (Shakirova *et al*., 2003), drought (Singh and Usha, 2003), heavy metal toxicity (Krantev *et al.,* 2008; Popova *et al.,* 2009).

The ameliorative effects of salicylic acid have been documented in inducing salt tolerance in many crops (El-Tayeb, 2005; Gunes *et al*., 2005; Stevens, 2006). Foliar application of salicylic acid exerted a significant effect on plant growth and metabolism when applied at physiological concentration and thus acted as one of the plant growth regulating substances (Kalarani *et al*., 2002).

Stimulation of growth after supplementation of SA has been reported in many plants, such as wheat (Shakirova *et al.,* 2003), soybean (Gutierrez-Coronado *et al.*,1998) and maize (Gunes *et al.*,2007) . It was found that SA-induced increase in growth could be related to SA-induced considerable enhancement in net photosynthetic rate under salt stress, particularly at 200 mg/l SA level (Noreen *et al.,* 2008). Salt induced high production of ROS can cause damages to mitochondria and chloroplasts (Apel *et al.,* 2004) and the efficiency of the antioxidative systems is correlated with tolerance to salt stress (Munns *et al.,* 2008) which increase plant tolerance to abiotic stress. However, SA cannot induce abiotic stress tolerance in all types of plants or in other words the effectiveness of SA in inducing stress tolerance depends upon type of species or concentration of SA applied (Arfan *et al.,* 2007). Biochemical studies have shown that plants under salt stress accumulate a number of metabolites, which are termed compatible solutes because they do not interfere with the plant metabolism and the accumulation of these solutes contribute to turgor maintenance in plants. Among them, the accumulation of low molecular weight solutes (compatible osmolytes), proline and glycinebetaine function as osmoprotectants. SA has been shown to ameliorate salt stress in seedlings of cucumber (Shim *et al.,* 2003), Brassica juncea and tomato (Molina *et al.,* 2002).

Jayalakshmi and her co workers (2010) investigated that the patent impact of SA on various areas of plant structure and function prompt many investigators to apply them in several crop plants aiming is control pattern of growth and development coupled with enhancement of systematic resistance against various hurtful agents which may appear in the surrounding environments. SA promotes some physiological processes and inhibiting others depending on its concentration, plant species, development stages and environmental conditions (Ding and Want, 2003; Mateo *et al.,* 2006). Salt induced high production of ROS can cause damages to mitochondria and chloroplasts and the efficiency of the antioxidative systems is correlated with tolerance to salt stress, which increase plant tolerance to several abiotic stress, including osmotic stress (Wang *et al.*, 2010) and heavy metal stress (Moussa and El-Gamel, 2010) and also influence a range of diverse processes in plants, including seed germination, stomatal closure, ion uptake and transport, membrane permeability, photosynthesis and plant growth rate (Aftab *et al.*, 2010). Patel *et al.* (2013) recently reported SA sustained antioxidant system under drought stress particularly in chickpea. Soybean is considered as a salt sensitive plant species (Lauchli, 1984). Hence, an experiment was conducted to determine whether exogenous applied salicylic acid (SA) could induce salt tolerance in soybean and to draw relationships between various biochemical parameters, osmolytes and antioxidative enzymes to elucidate mechanism associated with improved salinity tolerance in soybean.

## MATERIALS AND METHODS

### Plant Material, Treatment, and Plant Growth Conditions

A net house experiment was conducted in the Department of Plant Physiology, Institute of Agricultural Sciences, Banaras Hindu University,Varanasi by taking soybean genotype JS - 335 obtained from Department of Genetics and Plant Breeding, JNKVV Jabalpur (M.P.). 3-4 seeds per pot were surface sterilized and sown in plastic pots. The experiments were carried out during *kharif* season in july with the following treatment combinations: $T_1$-Control, $T_2$- 50 mM NaCl, $T_3$- 100 mM NaCl, $T_4$- Control + 100 ppm SA, $T_5$- 50 mM NaCl + 100 ppm SA, $T_6$- 100 mM NaCl + 100 ppm SA, $T_7$- Control + 200 ppm SA, $T_8$- 50 mM NaCl + 200 ppm SA and $T_9$- 100 mM NaCl + 200 ppm SA. Each treatment consisted of three replications in a complete randomized design (CRD). Two different concentrations of NaCl *viz.* 50 mM and 100 mM was applied in the soil by checking the EC of the soil and foliar spray of salicylic acid was done at 100 and 200 ppm in morning hours. Sampling was done at 45, 60 and 75 DAS for the following biochemical estimations.

### Sampling and Analysis

The plant samples were analyzed at 45, 60 and 75 DAS for the changes in the accumulation of biochemicals. Total soluble sugar (mg $g^{-1}$ fresh weight) and starch content (mg $g^{-1}$ fresh weight) was estimated by Anthrone method (Dubois *et al.*, 1956). The proline content ($\mu$g $g^{-1}$ fresh weight) of the leaf sample was estimated according to Bates *et al.*, (1973). Catalase activity was measured according to the protocol of Abei *et al.*, (1984). Superoxide dismutase activity was assayed by the method of Dhindsa *et al.*, (1981). The total chlorophyll content was estimated as per methods described by Arnon (1949).

### Statistical Analysis

The data were taken in triplicates and analysed using completely randomized design and data were subjected to analysis of variance (ANOVA). Data were compared at $p < 0.01$ level. Standard error of mean were also calculated (Gomez and Gomez, 1984).

## RESULTS AND DISCUSSION

### Total chlorophyll content

SA showed prominent effects and exhibited manifold increase in the level of accumulation of defense related biomolecules and total chlorophyll under salt stress in net house conditions. The accumulation of total chlorophyll in the soybean leaves showed both increasing and decreasing trend as salinity level increased over control after 45 (Table 1), 60 (Table 2) and 75 (Table 3) days of sowing. Not much significant differences were observed on application of foliar application of salicylic acid. The chlorophyll content of soybean leaves was increased due to application of salicylic acid (Khan *et al.*, 2003). Higher chlorophyll content in response to application of salicylic acid might account for synthesis of more carbohydrates in treated plants. However, reduction in chlorophyll content under influence of salicylic acid in certain crops like *Vigna mungo* has been reported by Anandhi and Ramanujam, (1997). The reduction of total chlorophyll content was probably related to the enhanced activity of the enzyme chlorophyllase. The role of SA in the chlorophyll levels was mentioned by Abreu and Munne-Bosch (2009) who revealed that SA deficiency is associated with reduced damage to the photosynthetic apparatus as well as chlorophyll levels.

Babar *et al.* (2014) reported that the growth medium salinity caused a marked reduction in photosynthetic pigments including chlorophyll a and chlorophyll b in fenugreek. The decrease in chlorophyll content in salt-affected fenugreek plants might be attributed to the possible oxidation of chlorophyll and other chloroplast pigments coupled with instability of the pigment protein complex under salt stress. Reduction in the chlorophyll content was mitigated by the foliar application of SA. Result of the present study are in agreement with the findings of earlier researchers where salt-induced reduction in the chlorophyll contents was alleviated by the foliar application of salicylic acid in crops such as wheat, mung bean, and tomato. Similar trends have been reported by Fahad and Bano, (2012) who revealed that salt stress significantly decreased the total chlorophyll content of leaves of maize plant.

### Sugar and starch content

Quantitative profile of total soluble sugar in soybean plants varied significantly within the plants treated with or without SA in comparison to untreated (control) under salinity stress after 45, 60 and 75 days of sowing (Table 1,2 and 3, respectively). Sugar content of salt stressed soybean cultivar increased with increasing salinity stress. It can be concluded that salicylic acid application at 100 ppm concentration may result in ameliorating salinity stress, but only upto a certain level of NaCl stress. Accumulations of carbohydrates such as sugars (e.g., glucose, fructose, fructans, and trehalose) and starch occur under salt stress. The major role played by these carbohydrates in stress mitigation involves osmoprotection,

**Table 1: Effect of salicylic acid in soybean at different salinity levels at 45 DAS on chlorophyll, sugar, starch, proline, superoxide dismutase and catalase**

| Treatment | Chlorophyll (mg $g^{-1}$ fresh weight) | | Sugar (mg $g^{-1}$ fresh weight) | | Starch (mg $g^{-1}$ fresh weight) | | Proline (µg $g^{-1}$ fresh weight) | | Superoxide dismutase (EU $g^{-1}$fresh weight) | | Catalase (EU $g^{-1}$ fresh weight) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| T1 | 0.53 | | 15.37 | | 45.39 | | 13.06 | | 1.96 | | 12.83 | |
| T2 | 0.60 | | 9.78 | | 52.20 | | 22.56 | | 2.56 | | 12.00 | |
| T3 | 0.53 | | 18.07 | | 71.20 | | 26.78 | | 2.95 | | 7.16 | |
| T4 | 0.63 | | 15.00 | | 31.12 | | 25.16 | | 2.10 | | 8.66 | |
| T5 | 0.79 | | 12.04 | | 72.47 | | 29.64 | | 1.92 | | 14.66 | |
| T6 | 0.65 | | 9.40 | | 75.79 | | 77.22 | | 2.79 | | 5.66 | |
| T7 | 0.26 | | 5.64 | | 40.25 | | 31.46 | | 2.54 | | 21.66 | |
| T8 | 0.80 | | 6.39 | | 88.72 | | 44.10 | | 3.05 | | 6.00 | |
| T9 | 0.77 | | 19.51 | | 44.13 | | 32.96 | | 3.08 | | 9.33 | |
| | SEm± | LSD** | SEm± | LSD** | SEm± | LSD** | SEm± | LSD** | SEm± | LSD** | SEm± | LSD** |
| Salinity | 0.060 | 0.103* | 1.430 | 2.426NS | 4.612 | 7.825NS | 3.786 | 6.423NS | 0.228 | 0.387NS | 1.248 | 2.117* |
| Salicylic acid | 0.060 | 0.103NS | 1.430 | 2.426NS | 4.612 | 7.825NS | 3.786 | 6.423NS | 0.228 | 0.387NS | 1.248 | 2.117NS |
| S* SA Int. | 0.105 | 0.178NS | 2.477 | 4.202NS | 7.989 | 13.553NS | 6.557 | 11.125NS | 0.395 | 0.671NS | 2.161 | 3.667* |

*Denotes significantly different at CD 1% and NS denotes non significant values. **p = 0.01

**Table 2: Effect of salicylic acid in soybean at different salinity levels at 60 DAS on chlorophyll, sugar, starch, proline, superoxide dismutase and catalase**

| Treatment | Chlorophyll (mg $g^{-1}$ fresh weight) | | Sugar(mg $g^{-1}$ fresh weight) | | Starch(mg $g^{-1}$ fresh weight) | | Proline(µg $g^{-1}$ fresh weight) | | Superoxide dismutase (EU $g^{-1}$fresh weight) | | Catalase (EU $g^{-1}$ fresh weight | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| T1 | 0.21 | | 25.97 | | 109.03 | | 86.26 | | 2.60 | | 26.33 | |
| T2 | 0.45 | | 16.94 | | 88.05 | | 154.96 | | 3.38 | | 34.16 | |
| T3 | 0.17 | | 25.54 | | 105.13 | | 121.16 | | 2.69 | | 70.00 | |
| T4 | 0.35 | | 20.32 | | 66.26 | | 100.18 | | 2.91 | | 60.00 | |
| T5 | 0.16 | | 20.49 | | 184.23 | | 141.05 | | 3.00 | | 64.16 | |
| T6 | 0.23 | | 24.46 | | 165.31 | | 120.12 | | 1.52 | | 47.00 | |
| T7 | 0.55 | | 19.20 | | 192.26 | | 204.15 | | 1.64 | | 54.16 | |
| T8 | 0.38 | | 17.69 | | 149.09 | | 197.74 | | 1.95 | | 49.50 | |
| T9 | 0.36 | | 15.05 | | 139.75 | | 155.22 | | 1.54 | | 83.83 | |
| | SEm± | LSD** | SEm± | LSD** | SEm± | LSD** | SEm± | LSD** | SEm± | LSD** | SEm± | LSD** |
| Salinity | 0.060 | 0.103NS | 2.290 | 3.884NS | 8.385 | 14.225NS | 12.020 | 20.393NS | 0.226 | 0.452S | 2.840 | 4.818* |
| Salicylic acid | 0.060 | 0.103NS | 2.290 | 3.884* | 8.385 | 14.225* | 12.020 | 20.393* | 0.226 | 0.452* | 2.840 | 4.818* |
| S*SA Int. | 0.105 | 0.178NS | 3.966 | 6.728* | 14.523 | 24.639* | 20.820 | 35.321NS | 0.461 | 0.783NS | 4.919 | 8.345* |

* Denotes significantly different at CD 1 % and NS denotes non significant values. **p = 0.01

carbon storage, and scavenging of reactive oxygen species. It was observed that salt stress increases the level of reducing sugars (sucrose and fructose) within the cell in a number of plants belonging to different species. Also, increase in soluble sugar level by application of salicylic acid in salt stressed maize plants by Khodary, (2004) has been reported. The soluble sugar in oat organ plant (root and bud) increased with increasing NaCl (El-Tayeb, 2005). With salicylic acid, the leaves fill up more soluble sugar and proline (Szepesi, 2006). The increasing of photosynthetic carbohydrate is a signal for water deficiency tolerance. The high carbohydrate concentration with its role to reduce water potential helps to prevent oxidative losses and protein structure maintenance during water shortage. The use of salicylic acid could activate the consumption of soluble sugar metabolism by increasing osmotic pressure. It is also supposed that salicylic acid treatment deranges the enzymatic system of polysaccharide hydrolysis (Khodary, 2004). The salt stress in soybean varieties decreased sugar level (EL-Samad and Shaddad, 1997).

Significantly higher soluble sugar content in leaves was found in maize plants of saline field, where salicylic acid further augmented the sugar production under salinity stress. SA induced increase was greater than that of without SA application under saline condition (Fahad and Bano, 2012). Gupta and Huang (2014) reported that sugar content was found to increase in tomato (*Solanum lycopersicum*) under salinity due to increased activity of sucrose phosphate synthase. Salicylic acid application at 100 ppm, shows higher value of starch in combating the salinity stress at both 50 and 100 mM concentrations. In case when salicylic acid applied at concentration of 200 ppm, significant differences were observed at 45, 60 and 75 days of sowing (Table 1, 2 and 3 respectively). In rice roots it has been observed that starch content decreased in response to salinity while it remained fairly unchanged in the shoot. Decrease in starch content and increase in reducing and nonreducing sugar content were noted in leaves of *Bruguiera parviflora*.

### Proline

Results also revealed that significant changes were recorded for proline content at 45, 60 and 75 days of sowing (Table 1, 2 and 3, respectively). In respect to proline content in salt stressed soybean plants, application of salicylic acid at concentration of 200 ppm showed better response significantly in alleviating salt stress at salinity levels of 50 mM concentration when compared with only 50mM NaCl stressed plants. The results obtained by this study also supports the earlier reported ones, in the mechanism that accumulation of compatible

**Table 3: Effect of salicylic acid in soybean at different salinity levels at 75 DAS on chlorophyll, sugar, starch, proline, superoxide dismutase and catalase**

| Treatment | Chlorophyll (mg $g^{-1}$ fresh weight) | Sugar (mg $g^{-1}$ fresh weight) | Starch(mg $g^{-1}$ fresh weight) | Proline ($\mu$g $g^{-1}$ fresh weight) | Superoxide dismutase (EU $g^{-1}$fresh weight) | Catalase ( EU $g^{-1}$ fresh weight) |
|---|---|---|---|---|---|---|
| T1 | 0.27 | 24.86 | 81.58 | 341.00 | 2.12 | 4.50 |
| T2 | 0.25 | 38.77 | 57.03 | 265.00 | 2.14 | 8.50 |
| T3 | 1.90 | 33.12 | 126.70 | 265.33 | 3.00 | 3.66 |
| T4 | 0.19 | 22.20 | 125.19 | 352.66 | 2.39 | 2.16 |
| T5 | 0.35 | 33.77 | 96.30 | 274.66 | 2.90 | 6.00 |
| T6 | 0.35 | 34.04 | 123.19 | 332.33 | 2.88 | 4.00 |
| T7 | 0.21 | 32.96 | 128.71 | 303.00 | 2.84 | 5.66 |
| T8 | 0.06 | 49.52 | 76.64 | 479.00 | 3.79 | 8.66 |
| T9 | 0.23 | 25.97 | 73.28 | 288.23 | 7.04 | 6.33 |
| | SEm± LSD** | SEm± LSD** | SEm± LSD** | SEm± LSD** | SEm± LSD** | SEm± LSD** |
| Salinity | 0.106 0.181* | 2.996 5.084* | 4.968 8.428NS | 22.099 37.491NS | 0.341 0.579* | 0.564 0.958* |
| Salicylic acid | 0.106 0.181NS | 2.996 5.084* | 4.968 8.428NS | 22.099 37.491NS | 0.341 0.579* | 0.564 0.958* |
| S* SA Int. | 0.184 0.313NS | 5.190 8.805* | 8.605 14.599* | 38.277 64.936* | 0.591 1.002* | 0.978 1.659NS |

*Denotes significantly different at CD 1 % and NS denotes non significant values, **p = 0.01

solutes. Proline, an amino acid besides acting as a cytoplasmic osmoticum, may function as a carbon and nitrogen source for post stress recovery and growth, as a stabilizer for membrane and protein synthesis machinery, as a scavenger of free radicals, as a sink for energy to regulate redox potential and also serves to protect the protein against denaturation. Proline accumulation was proposed to be associated with tolerance to osmotic and saline stress. Its concentration increases by either SA foliar spraying or salt stress. Shakirova *et al*. (2003) reported that wheat seedlings accumulated large amounts of proline under salinity stress which provided enhanced tolerance against salinity stress (Yusuf *et al*., 2008). It is well known that plants need more energy under saline soil environment. Extra energy could be provided by increased sugar, protein and proline accumulation which is energy rich compounds (Banaras *et al*., 2004). Interestingly, one molecule of proline produces 30 ATP molecules that help the plants to overcome, in part, the stressful environmental conditions. It can be proposed from this experiment that increased proline in salicylic acid treated plants might be due to reduced breakdown of proteins and enhanced incorporation of individual amino acid into proteins. El Samad and Shaddad (1997) concluded that the tolerance of salinity of soybean varieties was related to the accumulation of soluble protein, amino acids, proline, K and Ca and the sensitivity was associated with a decrease in water, sugar, protein, K and Ca.

Transcriptions factors such as OsNAC5 and ZFP179 showed an upregulation under salinity stress, which may regulate the synthesis and accumulation of proline. Gupta and Huang (2014) reported that the proline supplements enhanced salt tolerance in olive (*Olea europaea*) by amelioration of some antioxidative enzyme activities, photosynthetic activity, and plant growth and the preservation of a suitable plant water status under salinity conditions. It has also been reported that proline improves salt tolerance in *Nicotiana tabacum* by increasing the activity of enzymes involved in antioxidant defense system. Asadi *et al*. (2013) showed that priming with SA caused a considerable increasing in proline content in chickpea under salt stress.

***Antioxidative enzymes***

The superoxide dismutase (SOD) activity proved highest in plants treated with SA and under higher level of salt as compared to untreated one at 45, 60 and 75 days of sowing (Table 1, 2 and 3, respectively). Similarly, at 100 mM stress level, 200 ppm of foliar spray superoxide dismutase antioxidant enzyme resulted in higher activity only at a higher level of dose of salicylic acid *i.e.* 200 ppm in alleviating the salinity stress of both the salinity level of 50 and 100 mM NaCl significantly. SOD is the major superoxide scavenger and provides a first line of defense against the cellular due to environmental stress. One possible mechanism of salt tolerance in soybean is to elevate the contents and activities of various antioxidative components in order to restore the oxidative balance and minimize the cellular damage by secondary oxidative stress. It is also proposed that soybean which is considered as moderately salt tolerant might have inadequate ROS scavenging system, in addition to other tolerance mechanism, to cope with stress.

The increase in the activity of antioxidant enzyme following SA application could be the indicator of buildup of a protective mechanism to reduce oxidative damage induced by salt stress. Thus, it is in context that salicylic acid ameliorates the harmful effects of salinity stress showed higher activity. It has been suggested that salinity causes oxidative stress by inhibition of the carbon dioxide assimilation, exposing chloroplasts to excessive excitation energy, which in turn increases the generation of ROS from triplet chlorophyll (Gossett *et al*., 1994). Catalase seems to be a key enzyme in salicylic acid induced stress tolerance since it was shown to bind salicylic acid in vitro (Chen *et al*., 1993). Similar pattern was also recorded in case of catalase activities in the soybean at 45, 60 and 75 days of sowing (Table 1, 2 and 3, respectively). The changes in CAT may vary according to the intensity of stress, time of assay, after stress and induction of new isozyme. Catalase activity increases during salinity stress and application of salicylic acid at 100 and 200 ppm level, proved to be helpful in alleviating the harmful effect of salinity at 50 mM NaCl level, by increasing the activity of antioxidant enzyme catalase. The increase in CAT activity may be due to the fact that $H_2O_2$ is one of the crucial ROS produced in response to different environmental stresses inducing salt and ionic stress and this $H_2O_2$ might act act as an inducer of its scavenging enzymes like CAT and other enzymes which in turn result in lowering the endogenous levels of $H_2O_2$.

Fahad and Bano (2012) reported that the saline condition resulted in significantly higher superoxide dismutase activity of leaves in maize plants. The SA treatment in plants grown in saline field had increased SOD activity. The SA treatment to plants of saline field showed decrease in catalase activity. They also investigated and reported that SA improved the antioxidant system of maize plants by increasing the SOD activities of leaves. Antioxidant metabolism, including antioxidant enzymes and nonenzymatic compounds, play critical role in detoxifying ROS induced by salinity stress. Salinity tolerance is positively correlated with the activity of antioxidant enzymes, such as superoxide dismutase (SOD) and with the accumulation of nonenzymatic antioxidant compounds (Gupta and Huang, 2014).

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