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INFLUENCE OF SALICYLIC ACID PRE-TREATMENT ON WATER STRESS AND ITS RELATIONSHIP WITH ANTIOXIDANT STATUS IN *GLYCINE MAX*

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ABSTRACT

The combined effect of salicylic acid (SA) (100, 200 and 400 ppm) and water stress (waterlogging and drought) on growth, reactive oxygen species generation and activities of enzymatic and non-enzymatic antioxidants were studied in soybean (*Glycine max* L. Merr.) leaves.The results proved that the interaction of salicylic acid with water stress significantly increased total protein content and decreased reactive oxygen species (superoxide anion radical and hydrogen peroxide) in soybean leaves. Water stress also motivated enzymatic (superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR)) and non-enzymatic (carotenoids, ascorbic acid, nonprotein thiol and proline) activity, while they had a declining trend as a consequence of increasing SA level. It showed prominent role of SA and a sign of oxidative damage in experimental models. . Further investigation to evaluate long term water stress effects is recommended.

KEYWORDS: Catalase, *Glycine max*, Reactive oxygen species, Salicylic acid, Superoxide dismutase, Water stress,

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INTRODUCTION

Salicylic acid (SA) is known as an important signal molecule or modulating plant responses to environmental stresses[1]. Salicylic acid (SA) is a naturally occurring plant hormone, influences various physiological and biochemical functions in plants. It can act as an important signaling molecule and has diverse effects to tolerate to biotic and abiotic stresses[2, 3]. Its role in plant tolerance to abiotic stresses such as ozone, heat, heavy metal and osmotic stress has been reported by several authors[4,5,6,7]. It can activate gene expression and influence a variety of signaling mechanisms in plant defense[8]. SA plays an important role in the defense response to environmental stresses in many plant species[9]. First plant response to waterlogging is the reduction in stomata conductance[10]. Plants exposed to flooding stress exhibit increased stomata resistance as well as, limited water uptake leading to internal water deficit[11]. In addition, low levels of O_2 may decrease hydraulic conductivity due to hampered root permeability[12]. Oxygen deficiency generally leads to the substantial decline in net photosynthetic rate[13]. This decrease in transpiration and photosynthesis is attributed to stomata closure[14]. However, other factors such as reduced chlorophyll contents, leaf senescence and reduced leaf area are also held responsible for decreased rates of photosynthesis[15]. In this context, Yordanova et al.[16] reported fast stomata closure in barley plants when subjected to flooding conditions. Drought is one of the most limiting factors for plant survival since it regulates growth and development and limits plant productivity. The effect of drought varies with the variety, degree and duration of stress and the growth stage of the plant. Water deficits cause much lower water potential in soybean during the reproductive stage accompanied by increase in leaf stomatal resistance than the vegetative stage[17]. The resulting effect is a reduction in carbon assimilation and subsequent biomass production. In several plants, growth and yield are slightly affected at the vegetative stage but drastically reduced at the reproductive stage[18]. Despite the fact that oxygen is important for life on earth, its reduction by any means could result in the production of reactive oxygen species (ROS) perturbing several cellular metabolic processes of plants[19]. Lethal reactive oxygen species include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^\cdot$). Singlet oxygen ($_1O^2$) generated due to the reaction of oxygen with excited chlorophyll, is also considered as potential ROS[20]. These ROS are extremely reactive in nature and induce damage to a number of cellular molecules and metabolites such as proteins, lipids, pigments, DNA etc[21]. ROS are also produced in plants under normal conditions or non stressed conditions but their concentration is very low. All the plants have the ability to detoxify the adverse effects of ROS by producing different types of antioxidants. Generally, antioxidants are categorized into enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include ascorbate peroxidase (APX),superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), glutathione reductase (GR), whereas, ascorbic acid, glutathione, tocopherols and carotenoids are included in non-enzymatic antioxidants[22].The aim of the present study was to investigate the ameliorative effect of foliar application of salicylic acid on non enzymatic and enzymatic antioxidants water stressed *Glycine max*.

MATERIALS AND METHODS

Growth conditions and Plant material

The experiment was carried out in greenhouse ambient of school of Forestry and Environmental Science at Sam Higginbottom Institute of Agriculture, Technology and Sciences (SHIATS) (Deemed-to be-University), Allahabad-211007, India, during the months of July to October of 2011. The plants grown in greenhouse ambient under natural conditions day/night (minimum/maximum air temperature and relative humidity were: 22.4/37.6 ºC and 76 to 81%,respectively, as well as the average photoperiod was of 12 h of light and maximum

active photosynthetical radiation of 623 μmol^{-2} s^{-1} (at12:00 h). *Glycine max* seeds were collected from Genetics and Plant Breading department, SHIATS (Deemed-to be-University), Allahabad, India, were surface sterilized with 0.01 % aqueous solution of mercuric chloride followed by repeated washing with double distilled water (DDW). These seeds were sown in earthen pots (10 inches diameter) filled with sandy loam soil and farmyard manure (mixed in the ratio of 6:1) and lined in a green house. At 20 days stage, plants were sprayed with 100, 200 and 400 ppm of salicylic acid (SA). Each seedling was sprinkled thrice. The nozel of the sprayer was adjusted in such a way that it pumped out 1 ml in one sprinkle. Therefore, each plant received 3 ml of SA solution. After completing last treatment of SA, water stress (Drought and Waterlogging stress) was maintained. The experiments were allocated to eight groups as follows: T_0 (Normal irrigation),T_1 (Waterlogging control), T_2(Waterlogging + 100 ppm SA), T_3 (Waterlogging + 200 ppm SA), T_4 (Waterlogging + 400 ppm SA),T_5 (Drought control), T_6 (Drought + 100 ppm SA), T_7 (Drought + 200 ppm SA) and T_8 (Drought + 400 ppm SA). The plants were sampled at 10, 20 and 30 days after maintaining water stress to assess the following observations:

Protein estimation

Protein content in the plant extracts was determined according to Lowry et al[23]. One gram fresh leaves were homogenized with 10 ml phosphate buffer (1mM, pH 7.0). The homogenate was centrifuged at 8000 rpm for 30 minutes. The supernatant was used for protein estimation. Its 100 µl, and 200 µl of the aliquots were taken in triplicate for test and maintained to 500 µl by water, followed by the addition of 5 ml of reagent-C, (reagent-C: 95 ml of reagent-A mixed with 5 ml of reagent-B, Reagent-A: 2% sodium carbonate in 0.1 M NaOH, Reagent-B: 1% copper sulphate ($CuSO_4.5H_2O$), 2% potassium-sodium tartarate in ratio of 1:1 was also mixed properly andincubated for 10 min at room temperature. 500µl of 1N Folin-Ciocalteu's phenol reagent was mixed and vertexed quickly. This reaction mixture was incubated for 30 minutes at 37°C and its absorbance was recorded at λmax 660nm. The amount of protein was calculated by comparison with standard curve of BSA drawn under identical experimental conditions.

Measurements of ROS Production

Determination of Superoxide anion ($O_2^{\cdot -}$) production

Superoxide anion radicle production ($O_2^{\cdot -}$) rate in leaves were determined by the method utilized by Doke (1983). One gram leaves were placed in in a test tube and poured over with a solution containing 0.05 M PBS (pH 7.8), 0.05% nitroblue tetrazolium (NBT)and 10 mM NaN_3. After 5 minutes incubation in the dark, 2 ml of the solution was taken up from the tubes and heated at 90 ºC for 10 minutes, then the samples were cooled and absorbance was measured at 580 nm.

Determination of hydrogen peroxide (H_2O_2) production

Hydrogen peroxide production were determined by the method described by the Andreae[24]. The leaves were preincubated for 30 minutes in 3 ml of PBS (20 mM, pH 6.0) to remove preform H_2O_2, then incubated 3 ml of the same buffer containing 5 µM scopoletin and 3 µg ml^{-1} horseradish peroxidase in dark ness at 25 ºC on a shaker. The decrease in fluorescence (excitation: 340 nm, emission 455 nm) in the incubation medium was measured using reagent blanks as reference. Fluorescence was transformed into molar H_2O_2 concentration using a linear calibration curve.

Enzymatic antioxidants

Enzyme extraction

Leaves tissues (100mg FW) were homogenized in 4 ml 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA. The homogenate was centrifuged at 15 000 rpm, at 4 °C for 20 min. The supernatant was stored at -20 ◦C and used for the assay of enzyme activity.

Superoxide dismutase (SOD)

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by measuring the

inhibition of photochemical reduction of NBT[25]. The color was developed by adding the following reagents: 2.4 ml of 50mM potassium phosphate buffer solution (pH 7.8), 0.2 ml of 195 mM methionine, 0.1 ml of 0.3mM EDTA, 50µl enzyme extract, 0.2 ml of 1.125 mM NBT and 0.2ml of 60µM riboflavin. Reaction mixtures were illuminated for 15min at light intensity of 5000 lux and absorbance was recorded at 560 nm.

Catalase (CAT)

Catalase (CAT, EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 at 240nm for 1 min[26]. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 50µl of enzyme extract in a 3 ml volume. The enzyme activity was calculated using the extinction coefficient (39.4mM^{-1} cm^{-1}) and expressed as units (1µmol of H_2O_2 decomposed per minute) per mg protein.

Ascorbate peroxidase (APX)

Ascorbate peroxidase (APX, EC 1.11.1.11) activity measurement the reactive solution contained 50mMsodium phosphate buffer (pH 7.0), 0.5mM ascorbate, 0.1mM H_2O_2 and 10µl of enzyme extracts. The decrease in absorbance at 290 nm was read. Activity was calculated using the extinction coefficient (2.8mM^{-1} cm^{-1}). One unit of APX was defined as the amount of degrading 1µmol of ascorbate min^{-1} mg $protein^{-1}$ under the assay conditions[27].

Glutathione reductase (GR)

Glutathione reductase (GR) activity was determined according to Jablonski and Anderson [28]. The reaction mixture consisted of 10 mM GSSG, 1 mM EDTA, and 200 mM phosphate buffer. The supernatant was pre incubated at 25 °C for 5 min. The reaction was initiated by an addition of 1 mM NADPH, and the rate of oxidation of NADPH was monitored at 340 nm. The enzyme activity is expressed as µmol NADPH min^{-1} mg^{-1} protein.

Nitrate reductase (NR)

NR activity was determined by the method of Hageman and Hucklesby[29] with slight modification. For determination of NR activity100 mg of leaves were placed directly into 10 ml of incubation medium (300 mM KNO_3 as substrate in 1% isopropanol). The reaction was performed in the dark for 30 min in a water bath maintained at 30 ºC with constant shaking. NR activity was calculated as the amount of enzyme, which produced micromoles of nitrite g^{-1} fresh weight in 1 h. The amount of nitrite was determined spectrophotometrically at 540 nm.

Non enzymatic Antioxidants

Ascorbic acid

Ascorbic acid content was measured using a modified method of Davis and Masten[30]. Each leaf samples were extracted using1% of phosphate citrate buffer, pH 3.5 using chilled mortar and pestle. Then the homogenates was centrifuged at 10000 rpm at 4°C for 10 min. lastly, the supernatant was collected and used for further analysis. The supernatant was added with 1.72 mM 2,6-dichloroindophenol (2,6-DCPIP) in 3 ml cuvette and was measured at 518 nm immediately after mixing.

Carotenoids

Total carotenoids in the plant tissues were estimated according to the method by Jensen[31]. One gram of each sample were extracted with 80% methanol and centrifuged. The supernatants were concentrated to dryness. The residues thus obtained were dissolved in 15 ml of diethyl ether and after addition of 15 ml of 10% methanolic KOH, the mixture was washed with 5% ice-cold saline water to remove alkali. The collective saline washings were extracted with ether (3:15 v/v). The ether extract from both were mixed together followed by washing with cold water till alkali free. The alkali free ether extract was dried over anhydrous Na_2SO_4 for two hours in the dark. The ether extracts were filtered and its absorbance was measured at λmax 450 nm by using ether as blank.

Non Protein Thiol

Non protein thiols (NPT) were extracted by grinding 0.5 gm leaves tissue in 1 ml ice cold 5 % sulfosalicylic acid solution. After centrifugation at 10000 rpm at 4 ºC for 30 minutes, the supernatant were collected and immediately

assayed. NPT was measured by Ellaman's method[32]. Briefly, 300 µl of the supernatant was mixed with 1.2 ml of 0.1 M PBS (pH 7.6). After a stable absorbance reading of 412 nm was obtained, 25 µM 5,5-dithiobis-2-nitrobenzoic acid (DTNB) solution was added and the decrease in absorbance at 412 nm was monitored.

Proline

Proline content was determined based on the method of Bates et al.[33]. 100 mg of Leaf tissue was homogenized with 10 ml of 3% aqueous sulfosalicylic acid and centrifuged at 10,000 rpm for 10 min, 2ml of supernatant were mixed with 2ml of glacial acetic acid and 2ml of acid ninhydrin for 1 h at 100^0C. The developed colour was extracted in 4ml toluene and measured colourimetrically at 520nm. A standard curve with L-proline was used for the final calculations. Content of proline was expressed as mol g^{-1} FW (fresh weight).

Statistical Analysis

All the experiments were performed in triplicate. Values in the tables indicate mean values ± SD. Differences among treatments were analyzed by Two Way ANOVA with multiple observations, taking $p < 0.05$ as significant according to Fisher's multiple range test.

RESULTS

Multifarious antioxidant systems are very important for protecting cellular membranes and organelles from the damaging effects of active oxygen species. These include antioxidant enzymes, non enzymatic antioxidants.

Total protein content

The total protein content was significantly decreased in waterlogging (0.778±0.003mg/gm FW) and drought control (0.635±0.012 mg/gm FW) seedlings as compared to normal control (0.819±0.005 mg/gm FW) at 10 days after treatment (DAT). The foliar application of salicylic acid (SA) of different concentration (100, 200 and 400 ppm) increased total protein content. At 200 ppm of SA concentration the maximum total protein content was recorded in waterlogging and drought conditions with the mean values 0.955±0.005 and 0.918±0.007 mg/gm FW respectively at 10 DAT (Table 1). Later, it increased with increasing days as the similar trend.

Measurement of Reactive oxygen Species Production (ROS)

Superoxide anion ($O_2^{\cdot-}$) production

Superoxide anion ($O_2^{\cdot-}$) production content was increased in *Glycine max* plants under waterlogging and drought stress. Superoxide anion l ($O_2^{\cdot-}$) production content of leaves under water stress plants decreased significantly with increasing the level of salicylic acid (SA).At 200 ppm of SA application the maximum decrement of superoxide anion was recorded in waterlogging conditions with the mean values 10.21±0.295, 11.70±0.205, 14.99±0.192 mol min^{-1} mg^{-1}protein FW and in drought stress was 9.25±0.117, 11.39±0.175, 14.27±0.173mol min^{-1} mg^{-1} protein FW at 10 DAT, 20 DAT and 30 DAT respectively (Table 2). 200 ppm of SA concentration was recorded more decrement in superoxide anion production as compared to 100 and 400 ppm concentrations treated plants in both waterlogging and drought stress.

Hydrogen peroxide (H_2O_2) production

The hydrogen peroxide (H_2O_2) production was increased in waterlogging (7.84±0.080 µmol gm^{-1} FW) and drought control (6.21±0.182 µmol gm^{-1} FW) seedlings as compared to normal control (3.14±0.085 µmol gm^{-1} FW) at 10 DAT. The foliar application of (SA) of different concentration (100, 200 and 400 ppm) significantly decreased H_2O_2 production as compared to stress controls. TheH_2O_2 production was decreased 6.59±0.296, 4.64±0.200 and 5.12±0.100 µmol gm^{-1} FW under waterlogging at 100, 200 and 400 ppm SA concentration respectively as compared to waterlogged control (7.84±0.080 µmol gm^{-1} FW), whereas under drought stress H_2O_2 production was decreased at 100 ppm (5.04±0.142 µmol gm^{-1} FW), 200 ppm (2.96±0.125 µmol gm^{-1} FW) and 400 ppm (3.34±0.085 µmol gm^{-1} FW) concentration of SA as compared to drought control (6.21±0.182 µmol gm^{-1} FW) at 10 DAT (Table 3). Later, it decreased in the similar

manner with increasing days at 20 DAT and 30 DAT.

Enzymatic Antioxidants

Enzymatic antioxidants were strongly affected by water stress. In fact, this result indicated that oxidative stress is one of the main water stress consequences on *Glycine max* and SA has an ameliorative effect on this process. SA application may cause a temporary and low level of oxidative stress in plants, which acts as a hardening process, improving the antioxidative capacity of plants and helping to induce the synthesis of protective compounds.

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was increases on waterlogging control (118.31±2.080 Unit mg^{-1} FW) and drought control (110.30±1.10 Unit mg^{-1} FW) seedlings as compared with control plants (74.75±0.676 Unit mg^{-1} FW) at 10 DAT. SA application decreased specific activity of SOD along with increasing applied SA concentration under water stress conditions. At 200 ppm of SA application the maximum decrement in specific activity of SOD was recorded in waterlogging conditions with the mean values 90.71±0.525, 96.01±0.800, 103.61±0.451 Unit mg^{-1} FW and in drought condition were 81.66±0.431, 86.87±0.451, 93.16±0.650 Unit mg^{-1} FW as compared to respective controls at 10, 20 and 30 DAT (Table 4). Reduced SOD activity could be a symptom of decreased oxidative stress severity which could be a result of SA application.

Catalase (CAT)

In Table 5, the specific activity of catalase has been seen to decrease at each SA treatment (100, 200 and 400 ppm) as compared to water stressed plants. An effective significant decline in specific activity of catalase was observed at 200 ppm of SA concentration. At 200 ppm concentrations of SA exhibited significant decrement in specific activity of catalase in waterlogging stress were recorded 131.86±0.702, 141.0±0.721, 147.73±0.503 µmol min-1 mg-1 protein FW and in drought stress were recorded 127.73±0.611, 135.86±0.929, 142.80±0.916 µmol min^{-1} mg^{-1} protein FW as compared to respective stressed control at 10, 20 and 30 DAT respectively.

Ascorbate peroxidase (APX)

Specific activity of APX was also affected by the applied waterlogging and drought stress. APX activity was increased in waterlogging control (21.83±0.550 µmol min^{-1} mg^{-1} protein FW) and drought control (19.73±0.186 µmol min^{-1} mg^{-1} protein FW) seedlings as compared to control (5.35±0.396 µmol min^{-1} mg^{-1} protein FW). SA application had an additive effect on specific activity of APX and along with increasing SA level (100, 200 and 400 ppm), it was decreased. Treatments with different concentration of SA in waterlogging and drought stress seedlings caused maximum decrement in specific activity of APX with the mean values at 200 ppm 8.63±0.838, 11.92±0.170, 14.82±0.301 µmol min^{-1} mg^{-1} protein FW in waterlogging; 6.53±0.706, 9.26±0.216, 12.80±0.357 µmol min^{-1} mg^{-1} protein FW in drought stressed plants as compared to respective controls at 10, 20 and 30 DAT respectively (Table 6).

Glutathione reductase (GR)

Waterlogging and drought stress increased specific activity of glutathione reductase (GR) as compared to control. Like the previous, SA application leads to a lower GR activity on waterlogging and drought stressed seedlings. It could also be a result of reduced oxidative damage due to SA application and so this caused a decreased GR activity. Specific activity of GR was decreased when waterlogging and drought stressed plants treated with 100 (166.30±1.053, 156.36±0.763 µmol min^{-1} mg^{-1} protein FW), 200 (145.53±0.585, 141.96±2.318 µmol min^{-1} mg^{-1} protein FW) and 400 ppm (159.10±0.818, 153.26±1.069 µmol min^{-1} mg^{-1} protein FW) concentration of SA as compared to stressed control seedlings at 10 DAT. Specific activity of GR was decreased as the identical manner with increasing days at 20 DAT and 30 DAT (Table 7).

Nitrate reductase (NR)

Specific activity of Nitrate reductase (NR) was increased in waterlogging control (254.30±1.67 µmol NO_2 h^{-1} g^{-1} FW) and drought control (244.20±0.70 µmol NO_2 h^{-1} g^{-1} FW) seedlings as compared to normal control (191.81±0.43 µmol NO_2 h^{-1} g^{-1} FW) at 10 DAT. Specific activity of NR was significantly decreased under rising concentration of SA ranges from 100 to 400 ppm. At 200 ppm of SA concentration the maximum decrement was recorded in waterlogging and drought conditions with the mean values 210.70±1.76 and 198.83±0.70 µmol NO_2 h^{-1} g^{-1} FW respectively at 10 DAT (Table 8). Later, it increased as the similar trend with increasing days at 20 and 30 DAT.

Non enzymatic Antioxidants

Ascorbic acid

Seedlings under waterlogging and drought stress conditions showed a drastic increment on ascorbic acid content as compared to control. The average mean value of the ascorbic acid content of the control plants was 0.366±0.020 mg gm^{-1} FW, while the ascorbic acid content of 0.968±0.017 and 0.902±0.019 mg gm^{-1} FW were observed when the waterlogging and drought stress were imposed. Along with increasing SA concentration, ascorbic acid content was decrease in both stressed plants. There was a little decrease at 100 ppm SA on ascorbic acid content, but not significant. Maximum decrement was recorded at 200 ppm application of SA under waterlogging and drought stress were 0.528±0.040 and 0.621±0.012 mg gm^{-1} FW respectively at 10 DAT (Table 9). Ascorbic acid content was decreased as the identical manner with increasing days at 20 DAT and 30 DAT. It could also be a result of reduced oxidative damage due to SA application and so this caused decreased ascorbic acid content.

Carotenoids

There was a significant decrease in carotenoids content of *Glycine max* leaves under waterlogging control (0.281±0.0301 mg/gm FW) and drought control (0.173±0.0240 mg/gm FW) as compared to normal control (0.488±0.0906 mg/gm FW) at 10 DAT. The SA treatment under water stress (waterlogging and drought) condition resulted higher carotenoids content as compared to that of waterlogging and drought control. At 200 ppm of SA concentration the maximum carotenoids content was recorded in waterlogging conditions with the mean values 0.435±0.0296, 0.655±0.0440, 0.921±0.0265 mg/gm FW and under drought condition were 0.350±0.030, 0.568±0.050, 0.830±0.0350 mg/gm FW at 10 DAT, 20 DAT and 30 DAT respectively (Table 10).

Non protein thiol (NPT)

After waterlogging and drought stress, the levels of NP-SH in leaves of *Glycine max* seedlings increased as compared to control. SA application decreased NPT content along with increasing applied SA concentration under water stress conditions. At 200 ppm concentrations of SA exhibited significant decrement of non protein thiol in waterlogging stress were recorded 14.80±0.556, 17.30±0.631, 20.82±0.631 nmol gm^{-1} FW and in drought stress were recorded 14.19±0.490, 16.70±0.754, 18.93±0.576 nmol gm^{-1} FW as compared to respective stressed control at 10, 20 and 30 DAT respectively (Table 11).

Proline

According to present study leaf free proline content was increased significantly under waterlogging (19.18±0.752 mg gm^{-1} FW) and drought stress (17.18±0.375 mg gm^{-1} FW) control as compared to normal control (6.76±0.550 mg gm^{-1} FW) at 10 DAT. Proline content was significantly decreased under rising concentration of SA ranges from 100 to 400 ppm. At 200 ppm of SA concentration the maximum decrement was recorded in waterlogging and drought conditions with the mean values 10.38±0.535, 14.18±0.340, 16.91±0.330 mg gm^{-1} FW and 9.11±0.415, 12.71±0.579, 15.97±0.325 mg gm^{-1} FW at 10, 20 and 30 DAT respectively (Table 12).

Table 1
Effect of Salicylic acid (SA) on total protein content (mg gm^{-1} FW) of Glycine max under water stress.

Treatment	10 DAT	20 DAT	30 DAT
T_0	0.819±0.005	0.886±0.007	0.927±0.007
T_1	0.778±0.003	0.831±0.006	0.891±0.012
T_2	0.826±0.005	0.875±0.005	0.945±0.005
T_3	0.955±0.005	1.045±0.044	1.18±0.035
T_4	0.920±0.005	0.967±0.002	1.04±0.035
T_5	0.635±0.012	0.685±0.007	0.734±0.015
T_6	0.757±0.006	0.806±0.005	0.857±0.005
T_7	0.918±0.007	0.971±0.006	1.012±0.016
T_8	0.885±0.006	0.937±0.005	0.970±0.010

All values are mean ± standard deviation (n = 3). Means with different letters within a column are significantly different (P < 0.05).
SE due to Irrigation = 0.006 CD due to Irrigation = 0.013
SE due to Days = 0.008 CD due to Days = 0.016
SE due to SA levels = 0.010 CD due to SA levels = 0.021

Table 2
Effect of Salicylic acid (SA) on superoxide anion radical (O_2^-) production (mol min^{-1} mg^{-1} protein FW) in Glycine max under water stress.

Treatment	10 DAT	20 DAT	30 DAT
T_0	8.84±0.311	11.44±0.392	14.71±0.150
T_1	16.57±0.095	18.12±0.145	21.10±0.209
T_2	13.98±0.145	15.85±0.200	18.55±0.247
T_3	10.21±0.295	11.70±0.205	14.99±0.192
T_4	11.15±0.349	12.87±0.166	15.85±0.231
T_5	13.70±0.215	15.72±0.150	18.35±0.070
T_6	10.85±0.211	12.91±0.105	16.11±0.205
T_7	9.25±0.117	11.39±0.175	14.27±0.173
T_8	10.07±0.112	12.52±0.142	15.89±0.241

All values are mean ± standard deviation (n = 3). Means with different letters within a column are significantly different (P < 0.05).
SE due to Irrigation = 0.144 CD due to Irrigation = 0.286
SE due to Days = 0.176 CD due to Days = 0.351
SE due to SA levels = 0.227 CD due to SA levels = 0.452

Table 3
Effect of Salicylic acid (SA) on hydrogen peroxide (H_2O_2) production (µmol gm^{-1} FW) in Glycine max under water stress.

Treatment	10 DAT	20 DAT	30 DAT
T_0	3.14±0.085	4.92±0.175	6.84±0.170
T_1	7.84±0.080	9.70±0.175	10.94±0.367
T_2	6.59±0.296	8.16±0.185	9.78±0.265
T_3	4.64±0.200	6.10±0.160	8.23±0.115
T_4	5.12±0.100	6.73±0.085	9.03±0.101
T_5	6.21±0.182	7.91±0.135	9.95±0.170
T_6	5.04±0.142	6.69±0.153	9.06±0.191
T_7	2.96±0.125	4.34±0.132	6.84±0.072
T_8	3.34±0.085	4.72±0.140	7.14±0.131

All values are mean ± standard deviation (n = 3). Means with different letters within a column are significantly different (P < 0.05).
SE due to Irrigation = 0.088 CD due to Irrigation = 0.174
SE due to Days = 0.107 CD due to Days = 0.213
SE due to SA levels = 0.138 CD due to SA levels = 0.275

Table 4

Effect of Salicylic acid (SA) on specific activity of Superoxide dismutase (SOD) (unit mg^{-1} FW) in Glycine max under water stress.

Treatment	10 DAT	20 DAT	30 DAT
T_0	74.75±0.676	80.77±1.14	90.70±0.556
T_1	118.31±2.080	124.28±0.851	130.84±0.880
T_2	107.10±1.595	110.82±0.425	117.75±0.421
T_3	90.71±0.525	96.01±0.800	103.61±0.451
T_4	97.29±0.844	102.51±0.885	110.21±0.655
T_5	110.30±1.10	115.23±1.05	121.68±0.436
T_6	92.83±0.404	97.80±0.400	104.86±0.737
T_7	81.66±0.431	86.87±0.451	93.16±0.650
T_8	85.76±0.393	90.74±0.469	97.80±0.300

All values are mean ± standard deviation (n = 3). Means with different letters within a column are significantly different (P < 0.05).
SE due to Irrigation = 0.554 CD due to Irrigation = 1.101
SE due to Days = 0.875 CD due to Days = 1.741
SE due to SA levels = 0.678 CD due to SA levels = 1.348

Table 5

Effect of Salicylic acid (SA) on specific activity of Catalase (CAT) (µmol min^{-1} mg^{-1} protein FW) in Glycine max under water stress.

Treatment	10 DAT	20 DAT	30 DAT
T_0	121.83±0.763	128.90±0.458	133.80±0.871
T_1	158.40±0.916	163.61±0.520	167.50±0.360
T_2	153.63±0.971	159.41±0.202	164.10±0.888
T_3	131.86±0.702	141.0±0.721	147.73±0.503
T_4	141.90±1.479	149.56±0.450	157.40±1.113
T_5	150.66±1.026	156.96±1.721	160.86±1.137
T_6	144.60±0.602	149.23±0.850	153.90±0.818
T_7	127.73±0.611	135.86±0.929	142.80±0.916
T_8	137.01±0.561	143.80±0.400	148.31±1.208

All values are mean ± standard deviation (n = 3). Means with different letters within a column are significantly different (P < 0.05).
SE due to Irrigation = 0.436 CD due to Irrigation = 0.866
SE due to Days = 0.533 CD due to Days = 1.061
SE due to SA levels = 0.688 CD due to SA levels = 1.369

Table 6

Effect of Salicylic acid (SA) on specific activity of Ascorbate peroxidase (APX) (µmol min^{-1} mg^{-1} protein FW) in Glycine max under water stress.

Treatment	10 DAT	20 DAT	30 DAT
T_0	5.35±0.396	9.52±0.315	12.48±0.301
T_1	21.83±0.550	25.34±0.251	28.83±0.351
T_2	16.26±0.208	20.77±0.253	23.90±0.879
T_3	8.63±0.838	11.92±0.170	14.82±0.301
T_4	11.53±0.305	14.46±0.550	18.36±0.550
T_5	19.73±0.186	23.25±0.323	27.26±0.351
T_6	14.50±0.249	18.05±0.150	21.70±0.400
T_7	6.53±0.706	9.26±0.216	12.80±0.357
T_8	10.44±0.341	14.48±0.325	18.13±0.305

All values are mean ± standard deviation (n = 3). Means with different letters within a column are significantly different (P < 0.05).
SE due to Irrigation = 0.256 CD due to Irrigation = 0.509
SE due to Days = 0.313 CD due to Days = 0.624
SE due to SA levels = 0.405 CD due to SA levels = 0.806

Table 7

Effect of Salicylic acid (SA) on specific activity of Glutathione reductase (GR) (µmol min^{-1} mg^{-1} protein FW) in Glycine max under water stress.

Treatment	10 DAT	20 DAT	30 DAT
T_0	133.90±1.479	142.36±3.002	152.50±1.253
T_1	178.33±1.514	195.16±2.444	210.40±1.951
T_2	166.30±1.053	183.10±0.556	195.33±1.850
T_3	145.53±0.585	157.6±1.058	168.86±1.527
T_4	159.10±0.818	174.03±1.342	185.56±1.582
T_5	164.83±2.212	181.16±1.850	196.03±2.668
T_6	156.36±0.763	173.06±1.429	187.53±1.167
T_7	141.96±2.318	151.46±2.797	161.53±2.107
T_8	153.26±1.069	166.83±1.193	180.83±1.193

All values are mean ± standard deviation (n = 3). Means with different letters within a column are significantly different (P < 0.05).

SE due to Irrigation = 0.971 CD due to Irrigation = 1.931
SE due to Days = 1.189 CD due to Days = 2.365
SE due to SA levels = 1.535 CD due to SA levels = 3.053

Table 8

Effect of Salicylic acid (SA) on specific activity of Nitrate reductase (NR) (µmol NO_2 h^{-1} g^{-1} FW) in Glycine max under water stress.

Treatment	10 DAT	20 DAT	30 DAT
T_0	191.81±0.43	216.98±1.42	234.43±1.95
T_1	254.30±1.67	281.29±1.12	311.63±1.05
T_2	236.06±0.75	264.23±0.85	293.23±0.75
T_3	210.70±1.76	241.36±0.75	271.23±0.70
T_4	219.16±0.75	253.16±0.70	281.80±0.65
T_5	244.20±0.70	269.26±0.71	296.96±0.85
T_6	216.36±0.55	248.96±0.50	276.16±0.65
T_7	198.83±0.70	223.26±0.71	252.30±0.75
T_8	206.16±0.71	236.43±0.95	264.00±0.55

All values are mean ± standard deviation (n = 3). Means with different letters within a column are significantly different (P < 0.05).

SE due to Irrigation = 0.960 CD due to Irrigation = 1.980
SE due to Days = 1.176 CD due to Days = 2.339
SE due to SA levels = 1.518 CD due to SA levels = 3.020

Table 9

Effect of Salicylic acid (SA) on Ascorbic acid (mg gm^{-1} FW) in Glycine max under water stress.

Treatment	10 DAT	20 DAT	30 DAT
T_0	0.366±0.020	0.499±0.015	0.696±0.025
T_1	0.968±0.017	1.037±0.013	1.12±0.021
T_2	0.863±0.019	0.927±0.015	0.985±0.013
T_3	0.528±0.040	0.684±0.012	0.761±0.018
T_4	0.691±0.017	0.808±0.015	0.908±0.017
T_5	0.902±0.019	0.998±0.013	1.044±0.012
T_6	0.816±0.011	0.910±0.018	0.997±0.075
T_7	0.621±0.012	0.725±0.008	0.812±0.021
T_8	0.720±0.011	0.809±0.016	0.890±0.017

All values are mean ± standard deviation (n = 3). Means with different letters within a column are significantly different (P < 0.05).

SE due to Irrigation = 0.008 CD due to Irrigation = 0.0162
SE due to Days = 0.010 CD due to Days = 0.0198
SE due to SA levels = 0.012 CD due to SA levels = 0.0256

Table 10
Effect of Salicylic acid (SA) on carotenoids (mg gm^{-1} FW) of Glycine max under water stress.

Treatment	10 DAT	20 DAT	30 DAT
T_0	0.488±0.0906	0.709±0.0265	0.957±0.0365
T_1	0.281±0.0301	0.542±0.0420	0.822±0.0385
T_2	0.330±0.0140	0.637±0.0356	0.844±0.0329
T_3	0.435±0.0296	0.655±0.0440	0.921±0.0265
T_4	0.381±0.0165	0.613±0.0256	0.873±0.0214
T_5	0.173±0.0240	0.338±0.0386	0.555±0.0416
T_6	0.258±0.0240	0.490±0.0366	0.689±0.0250
T_7	0.350±0.030	0.568±0.050	0.830±0.0350
T_8	0.311±0.013	0.524±0.0240	0.741±0.029

All values are mean ± standard deviation (n = 3). Means with different letters within a column are significantly different (P < 0.05).

SE due to Irrigation = 0.012 CD due to Irrigation = 0.0240
SE due to Days = 0.014 CD due to Days = 0.0295
SE due to SA levels = 0.019 CD due to SA levels = 0.0380

Table 11
Effect of Salicylic acid (SA) on Non protein thiol (NPT) (nmol gm^{-1} FW) in Glycine max under water stress.

Treatment	10 DAT	20 DAT	30 DAT
T_0	13.23±0.735	15.05±0.616	18.51±0.700
T_1	22.75±0.676	26.56±1.12	30.76±0.616
T_2	20.30±0.458	23.40±0.70	26.30±0.631
T_3	14.80±0.556	17.30±0.631	20.82±0.631
T_4	16.89±0.490	19.16±0.662	23.22±0.740
T_5	20.55±0.568	24.02±0.682	28.21±0.509
T_6	18.23±0.601	20.47±0.551	23.64±0.538
T_7	14.19±0.490	16.70±0.754	18.93±0.576
T_8	15.96±0.404	18.29±0.655	21.48±0.760

All values are mean ± standard deviation (n = 3). Means with different letters within a column are significantly different (P < 0.05).

SE due to Irrigation = 0.147 CD due to Irrigation = 0.292
SE due to Days = 0.180 CD due to Days = 0.358
SE due to SA levels = 0.233 CD due to SA levels = 0.464

Table 12
Effect of Salicylic acid (SA) on proline (mg gm^{-1} FW) in Glycine max under water stress.

Treatment	10 DAT	20 DAT	30 DAT
T_0	6.76±0.550	9.70±0.200	12.02±0.301
T_1	19.18±0.752	22.41±0.625	27.32±0.669
T_2	15.73±0.440	19.78±0.354	23.35±0.518
T_3	10.38±0.535	14.18±0.340	16.91±0.330
T_4	12.86±0.274	17.42±0.531	20.51±0.423
T_5	17.18±0.375	20.38±0.538	24.65±0.538
T_6	13.44±0.540	17.25±0.597	20.31±0.443
T_7	9.11±0.415	12.71±0.579	15.97±0.325
T_8	10.96±0.185	14.79±0.400	17.83±0.366

All values are mean ± standard deviation (n = 3). Means with different letters within a column are significantly different (P < 0.05).

SE due to Irrigation = 0.149 CD due to Irrigation = 0.296
SE due to Days = 0.182 CD due to Days = 0.362
SE due to SA levels = 0.235 CD due to SA levels = 0.468

DISCUSSION

The present study indicated that SA act as plant growth regulators that can separately or together counteract waterlogging and drought induced oxidative stresses. According to Jaleel et al.[34] although, the effects of waterlogging and drought stress on growth and development of plants have been studied in a large-scale, still, the physiological and biochemical responses of plants to waterlogging and drought stress are not well understood. The foliar application of SA was beneficial in overpowering the adverse effects of waterlogging and drought stress. Overwhelming evidence showed that drought induces oxidative stress through the production of active oxygen species such as superoxide, H_2O_2, OH^-, and $^1O_2$[35]. They will then react to O_2 in the absence of other acceptors. Afterwards, antioxidative defense system was activated in response to oxidative stress. Furthermore, waterlogging and drought stress increased certain ROS production in leaves and induced lipid peroxidation in chickpea[36]. The reduction in the total soluble proteins in the plants under water stress (waterlogging and drought) is due to probable increase of the proteases enzyme activity, in which this proteases enzyme promote the breakdown of the proteins and consequently decrease the protein amount presents in the plant under abiotic stress conditions[37]. In inadequate conditions to the plant active the pathway of proteins breakdown, because the plant use the proteins to the synthesis of nitrogen compounds as amino acids that might auxiliary the plant osmotic adjustment[38]. Similar results on reduction in the proteins were found by Ramos et al.[39] investigating the effects of the water stress in *Phaseolus vulgaris*. *Glycine max* seedlings treated with SA accumulated less H_2O_2 content as compared to waterlogging and drought control plants. This suggests that SA may also play an important role in inducing tolerance to oxidative stress conditions in *Glycine max* which is in conformity with Agarwal et al.[40] in the case of wheat genotypes. Peroxidation of lipids was increased significantly in the *G. max* plants which were treated with waterlogging and drought stress. Exogenous application of SA, reduced water stress induced increase in the content of MDA. Nitric oxide decreases accumulation of hydroxyl in salt treated wheat leaves by eliminating O_2^- and H_2O_2[41] and also, SA decreases the content of MDA by inhibiting production of hydroxyl radical[42].

The coordinate function of antioxidant enzymes such as SOD, APX, catalase and GR helps in processing of ROS and regeneration of redox ascorbate and glutathione metabolites. The oxidative damage to cellular components is limited under normal growing conditions due to efficient processing of ROS through a well coordinated and rapidly responsive antioxidant system consisting of several enzymes and redox metabolites. Overall, activities of all the antioxidant enzymes increased under waterlogging and drought stress in *Glycine max* seedlings. These results are in agreement with findings of Habibi et al.[43] and Tohidi-Moghaddam et al.[44]. The mutual action of CAT and SOD converts the toxic $O_2^{\cdot-}$ and H_2O_2 into water and molecular oxygen, preventing the cellular injure under drought stress[45]. Thus, the necessity of SOD activity is reduced. Meloni et al.[46] found that SOD activity increased in cotton cultivars under salinity stress. Decreasing SOD activity due to SA application was also reported by Choudhury and Panda[47], when rice seeds were primed with SA treatment and exposed to oxidative damage. Reduced SOD activity could be a symptom of decreased oxidative stress severity which could be a result of SA application. Catalase is responsible for decomposition and detoxification of H_2O_2 in the Peroxisomes. The activity of this enzyme is sensitive to both drought and heat stresses[48]. It was confirmed that SA exogenous application could improve antioxidants activity in plants[49]. There was a transitory reduction on CAT activity as a result of SA exogenous treatment[50]. SA intensified APX activity in order to facilitate oxidative damage protection. Salicylic acid has an affinity to bind with the enzymes like APX and CAT[51] which are involved in ROS metabolism and redox homeostasis. Alteration in

this homeostasis leads to induction of a defense response in plants[52]. Increasing APX activity as a consequence of exogenous SA application was also reported by Krantev et al.[53]. Waterlogging and drought stress had the higher GR activity, but like the previous, SA application leads to a lower GR activity on water stressed seedlings. It could also be a result of reduced oxidative damage due to SA application and so this caused a decreased GR activity. The possible explanation for the concentration based effect of SA on NR activity is that NR activity was induced and/or prevention of enzyme degradation was prevented. Results indicated that concentrations of SA at 100 to 400 ppm might induce NR synthesis by mobilization of intracellular NO_3^-, and provide protection to *in vivo* NR degradation in absence of NO_3^-[54]. Fariduddin et al.[55] reported increased NR activity due reduced concentrations of SA while higher concentrations were observed to be inhibitory to NR activity in *Brassica juncea* Czern & Coss cv. Varuna. Carotenoids effectively quench singlet oxygen derived from primary photochemical reactions and hence a close correlation was found between the carotenoids contents of the leaves and the foliar biomass production of tomato genotypes under salt stress. The observed increase in carotenoid content of SA treated leaves of plants under water stress condition may indicate the better defense system induced by SA. The antioxidant property of NP-SH depends on the oxidation of –SH group of the tripeptide to disulfide form[56]. Generally, the plant exhibition of high amount of NP-SH during water stress indicates its ability to tolerate cellular ions load. The increased level of NP-SH may also be due to the stimulation of sulfate reduction pathway enzymes such as Adenosine-5'-phosphosulfate (APS) reductase and serine acetyl transferase[57]; while decreases observed at shoot level could possibly due to NP-SH consumption for glutathione (GSH) and phytochelatins (PCs) synthesis[58]. Present study suggests that foliar treatment with SA significantly improves plant tolerance to water stress by the enhancement of NP-SH amounts in shoots. This could be due to the SA role in alleviating water stress oxidative stress[59]. According to present study leaf free proline content was increased significantly under waterlogging and drought stress as compared to control. Plants adapt to water stress by changes in morphology, altered patterns of development and cellular metabolism. A number of these adaptive responses are associated with the accumulation of osmolytes like sugars and proline[60]. Application of SA (100, 200 and 400 ppm) to waterlogging and drought stress, increased leaf proline content as compared to waterlogging and drought control. In this regard, Hussain et al.[61] also found that waterlogging and drought stresses increased the free leaf proline and glycinebetaine (GB) of sunflower and were further increased by exogenous application of GB and SA. Umebese et al.[60] showed that proline content was only slightly increased at all stages of growth in water stressed tomato and amaranth plants.

CONCLUSION

Data presented in this study indicated that waterlogging and drought stresses could cause oxidative damage in *Glycine max* seedlings through excessive generation of ROS and foliar application of SA increased levels of waterlogging and drought stress tolerance in *Glycine max* seedlings. Plants treated with SA exhibited slight injury symptoms whereas those that were not treated with SA had moderate damage and lost considerable portions of their foliage. Antioxidant enzymes (SOD, CAT, APX and GR) activities and ascorbic acid carotenoids, NPT and proline levels increase in *glycine max* leaves, on the other hand foliar application of SA (especially 200 ppm) induced protection against drought stress via maintenance of membrane integrity by decline in MDA content and more increase in antioxidant enzymes activities as well as proline accumulation. Based on the obtained results, it may be concluded that, application of exogenous SA can be a method to decrease water stress damages to plants. However, the application dose of SA needs further investigation according to different plant species and different growth stages.

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