Application of salicylic acid increases contents of nutrients and antioxidative metabolism in mungbean and alleviates adverse effects of salinity stress

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Abstract

Salicylic acid (SA), a naturally occurring plant hormone, is an important signal molecule known to have diverse effects on biotic and abiotic stress tolerance. Its growth-promoting effect on various plants has been shown, but the information on the response of mungbean, an important leguminous plant, to SA application under salt stress is limited. Mungbean (*Vigna radiata* L.) cultivar Pusa Vishal plants grown with 50 mM NaCl were sprayed with 0.1, 0.5, or 1.0 mM SA and basic physiological processes were studied to substantiate our understanding of their role in tolerance to salinity-induced oxidative stress and how much such processes are induced by SA application. Treatment of plants with 0.5 mM SA resulted in a maximum decrease in the content of Na^+, Cl^-, H_2O_2, and thiobarbituric acid reactive substances (TBARS), and electrolyte leakage under saline conditions compared to the control. In contrast, this treatment increased N, P, K, and Ca content, activity of antioxidant enzymes, glutathione content, photosynthesis, and yield maximally under nonsaline and saline conditions. The application of higher concentration of SA (1.0 mM) either proved inhibitory or was of no additional benefit. It was concluded that 0.5 mM SA alleviates salinity-inhibited photosynthesis and yield through a decrease in Na^+, Cl^-, H_2O_2, and TBARS content, and electrolyte leakage, and an increase in N, P, K, and Ca content, activity of antioxidant enzymes, and glutathione content.

Introduction

Salinity is one of the major stresses in arid and semi-arid regions causing adverse effects at physiological, biochemical,[1] and molecular levels,[2] limiting crop productivity.[1,3] Salt stress can disturb growth and photosynthetic processes by causing changes in the accumulation of Na^+, Cl^-, and nutrients, and disturbance in water and osmotic potential.[4] The increasing concentration of Na^+ and Cl^- in the rooting medium suppresses the uptake of essential nutrients N, P, K, and Ca,[5] and alters ionic relationships.[6] Salt stress also generates reactive oxygen species (ROS) causing oxidative stress on plants. These ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and singlet oxygen ($^1O_2$) can damage chloroplasts[7] and reduce photosynthesis, involving stomatal behavior and inhibition of photochemical processes,[8] and disturbance in homeostasis of Na^+ and Cl^- ions and essential mineral nutrients.[9] The decrease in photosynthesis may lead to reduction in growth and yield. Plants operate several mechanisms to counteract the adverse effects of salt. These mechanisms may be enhanced by the application of chemicals to the plants.[4] One such mechanism is the activation of an antioxidant enzyme system, which may be influenced by the interaction of plant growth regulators and salt. Plants containing high activities of antioxidant enzymes have shown considerable resistance to the oxidative damage caused by ROS.[7,10-11] The antioxidant enzyme system constitutes superoxide dismutase (SOD, EC 1.15.1.1) as the primary step of cellular defense. It dismutates superoxide ions (O_2^-) to H_2O_2 and O_2. Further, the accumulation of H_2O_2 is restricted by the action of the ascorbate-glutathione cycle, where ascorbate peroxidase (APX, EC 1.11.1.11) reduces it to H_2O. The final step is catalyzed by glutathione reductase (GR, EC 1.6.4.2), which catalyzes the NADPH-dependent reaction of oxidized glutathione (GSSG) to reduced glutathione (GSH).[12]

Salicylic acid (SA), a naturally occurring plant hormone, acts as an important signaling molecule in plants, and has diverse effects on tolerance to abiotic stress.[13-15] Exogenous application of SA may participate in the regulation of physiological processes in plants, such as stomatal closure, ion uptake and transport,[16] membrane permeability,[17] and photosynthesis and growth.[18] Its role in abiotic stress tolerance such as ozone, UV-B, heat, heavy metal, and osmotic stress[19-22] has been reported. Studies on barley (*Hordeum vulgare*), maize (*Zea mays*), wheat (*Triticum aestivum*), bean (*Phaseolus vulgaris*), lentil (*Lens culinaris*), and sunflower (*Helianthus annuus*) suggest that SA may be used to alleviate salt stress.[21,23-28] In contrast, studies on *Zea mays*[29] and *Arabidopsis*[30] have shown an inhibitory effect of SA. In view of the contrasting reports on the effect of SA and limited literature available on leguminous crops, the research was undertaken on mungbean (*Vigna radiata*), an important crop grown in Asia and other parts of the world, to study the influence of SA application in the alleviation of salinity stress.

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Materials and Methods

Plant material and growth conditions

Plants of mungbean [*Vigna radiata* L. (Wilczek)] cultivar Pusa Vishal were raised from seeds planted in 15 cm-diameter earthen pots filled with acid-washed sand purified according to Hewitt.[31] Two plants were grown in a pot kept in a naturally illuminated greenhouse (photosynthetically active radiation (PAR) >900±22 μmol/m²/s and average temperature of 33±2°C) of the Department of Botany, Aligarh Muslim University, Aligarh, India. Plants in each pot were fed with 300 ml Hoagland nutrient solution containing 50 mM NaCl every alternate day and 200 mL of deionised water daily. The nutrient solution was replaced weekly. Salicylic acid was dissolved in absolute ethanol then added dropwise to water (ethanol/water: 1/1000, v/v).[32] Fifteen days after sowing (DAS), SA was applied on the foliage of 0 or 50 mM NaCl grown plants at a concentration of 0.1, 0.5, or 1.0 mM with a hand sprayer. A control group of plants was grown without NaCl and sprayed with deionized water. A surfactant teepol (0.5%) was added with the control and SA treatment solutions. The volume of the spray was 30 mL per pot. The treatments were arranged in a completely randomized block design and replicated three times.

Measurements

At 15 days after salicylic acid application (i.e. 30 DAS), the content of Na^+ and Cl^-, nutrients, H_2O_2, and thiobarbituric acid reactive substances (TBARS), electrolyte leakage, activity of antioxidant enzymes, and photosynthetic traits were determined from each treatment and replicate. At harvest (60 DAS), yield traits were recorded.

Content of Na^+ and Cl^- and nutrients

The determination of Na^+, Cl^-, N, P, K, and Ca was done in acid-peroxide digested oven-dried leaf samples. Na^+, K, and Ca were measured using a flame photometer (Khera-391: Khera Instruments, New Delhi) whereas Cl- was determined by potentiometric titration with $AgNO_3$. N and P were determined by using the methods of Lindner,[33] and Fiske and Subba Row,[34] respectively.

Content of H_2O_2 and thiobarbituric acid reactive substances, and electrolyte leakage

The content of H_2O_2 was determined following the method of Okuda *et al.*[35] Fresh leaf tissues (50 mg) were ground in ice-cold 200 mM perchloric acid. The reaction was started by the addition of peroxidase and increases in the absorbance were recorded at 590 nm for 3 min. The content of TBARS was measured according to the method described by Dhindsa *et al.*[36] For measuring electrolyte leakage, samples were thoroughly washed with sterile water, kept in closed vials with 10 mL deionized water and were incubated at 25ºC for 6 h using a shaker, and electrical conductivity (EC) was determined (C_1). Again samples were kept at 90ºC for 2 h and EC was obtained after attaining equilibrium at 25ºC (C_2). Electrolyte leakage was calculated using the formula: EC (%) = $(C_1/C_2) \times 100$.

Antioxidant enzymes

Leaves used for photosynthesis measurement were selected for the assay of antioxidant enzymes. Leaf tissue (200 mg) was homogenized with an extraction buffer containing 0.05% (v/v) Triton X-100 and 1% (w/v) polyvinylpyrrolidone in 100 mM potassium phosphate buffer (pH 7.0) using a chilled mortar and pestle. The homogenate was centrifuged at 15000 × g for 20 min at 4ºC. The supernatant obtained after centrifugation was used for the assay of SOD and GR enzymes. For the assay of APX, extraction buffer was supplemented with 2 mM ascorbate.

SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT), according to the methods of Beyer and Fridovich,[37] and Giannopolitis and Ries.[38] APX was determined according to Nakano and Asada[39] by the decrease in absorbance of ascorbate at 290 nm. GR activity was determined by the method described by Foyer and Halliwell[40] by monitoring the glutathione dependent oxidation of NADPH at 340 nm.

Glutathione content

The leaf tissue (200 mg) was homogenized in an ice bath in 1.0 mL of 5% 5-sulphosalicylic acid and the homogenate was centrifuged for 10,000 × g for 10 min. The supernatant was used for glutathione (GSH) estimation using 5,5'-dithio-2,2'-dinitrobenzoic acid (DTNB) by reading the absorbance at 412 nm, following the method of Anderson.[41]

Photosynthetic traits

The activity of CA in leaf was estimated in the leaves used for photosynthesis measurement according to the method described by Makino *et al.*,[42] determined following time-dependent reduction in pH 8.25-7.45 at 0-3°C. Net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and transpiration rate were measured in fully expanded, uppermost leaves of plants using an infrared gas analyzer (LiCOR-6200, Portable Photosynthesis System, Nebraska, USA). The measurements were done between 11h00-12h00 at light-saturating intensity and at atmospheric CO_2 concentration 365±5 µmol/mol. Water use efficiency was calculated as the ratio of photosynthesis to transpiration.

Yield traits

At harvest (60 DAS), pods were collected and counted. Pod length was measured on a meter scale. The number of seeds from each pod was counted. Seeds from a plant in each treatment were cleared, sun-dried, and weighed to compute seed yield per plant.

Data analysis

Data were statistically analyzed using SPSS, 10.0 for Windows. Standard error was calculated and analysis of variance (ANOVA) was performed on the data to determine the least significant difference (LSD) between treatment means with the level of significance at $P<0.05$.

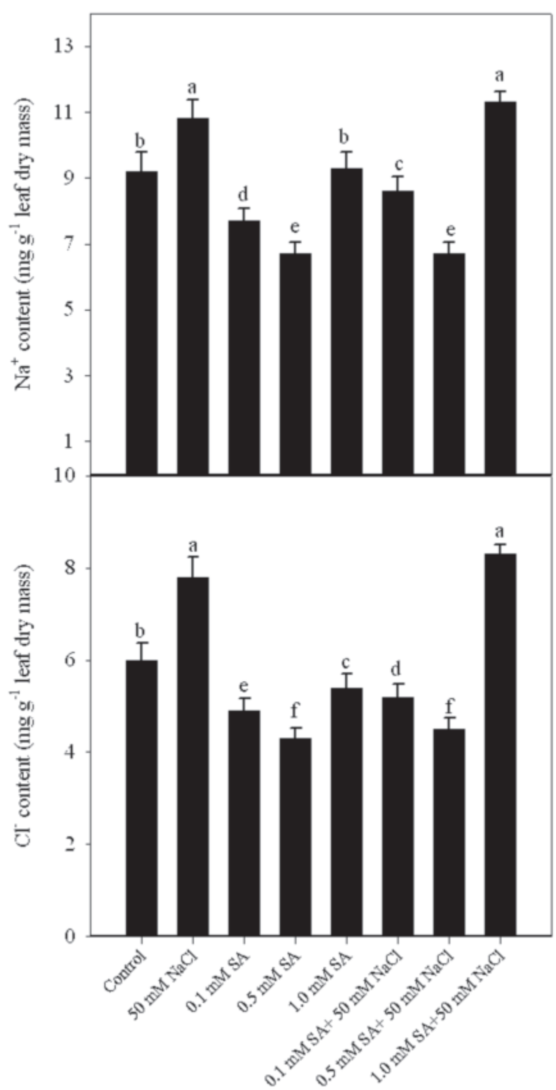

Figure 1. Effect of salicylic acid spray at 15 days after sowing (DAS) on Na^+ and Cl^- content of mungbean (*Vigna radiata* L.) plants grown with 0 or 50 mM NaCl at 30 DAS. Values are mean±SE. Data labeled by the same letter did not differ significantly at $P<0.05$.

Results

Content of Na^+, and Cl^- and nutrients

Plants grown with 50 mM NaCl exhibited an increase of 17.4% and 30.1% in Na^+ and Cl^- content, and decrease of 20.8%, 23.3%, 19.3%, and 18.2% in N, P, K, and Ca content, respectively, compared to the control (Figures 1 and 2). Under nonsaline conditions, SA application significantly decreased the content of Na^+ and Cl^-, and enhanced N, P, K, and Ca content, but the effect was more pronounced with 0.5 mM SA (Figures 1 and 2). The decrease in Na^+ and Cl^- content with 0.5 mM SA application was 27.2% and 28.3% with respect to the control, while this treatment resulted in the increase of N by 32.7%, P by 75.0%, K by 32.3%, and Ca by 43.6%, in comparison to the control. The content of Na^+ and Cl^- in plants treated with 0.1 mM SA and grown with 50 mM NaCl was less compared to the control. The application of 0.1 mM SA on plants grown with 50 mM NaCl increased the nutrients, content compared to the control. The application of 0.5 mM SA maximally reversed the effect of NaCl on the Na^+ and Cl^- content. This treatment (0.5 mM SA) also promoted N, P, K, and Ca content under salt stress. The content of Na^+ and Cl^- was reduced by 27.2% and 25.0% in comparison to the control with the application of 0.5 mM SA. The application of 0.5 mM SA resulted in an increase of N, P, K, and Ca content by 10.1%, 31.6%, 19.3%, and 19.1%, respectively, compared to the control. The application of 1.0 mM SA did not alleviate the negative effects of NaCl on ions and nutrients, content.

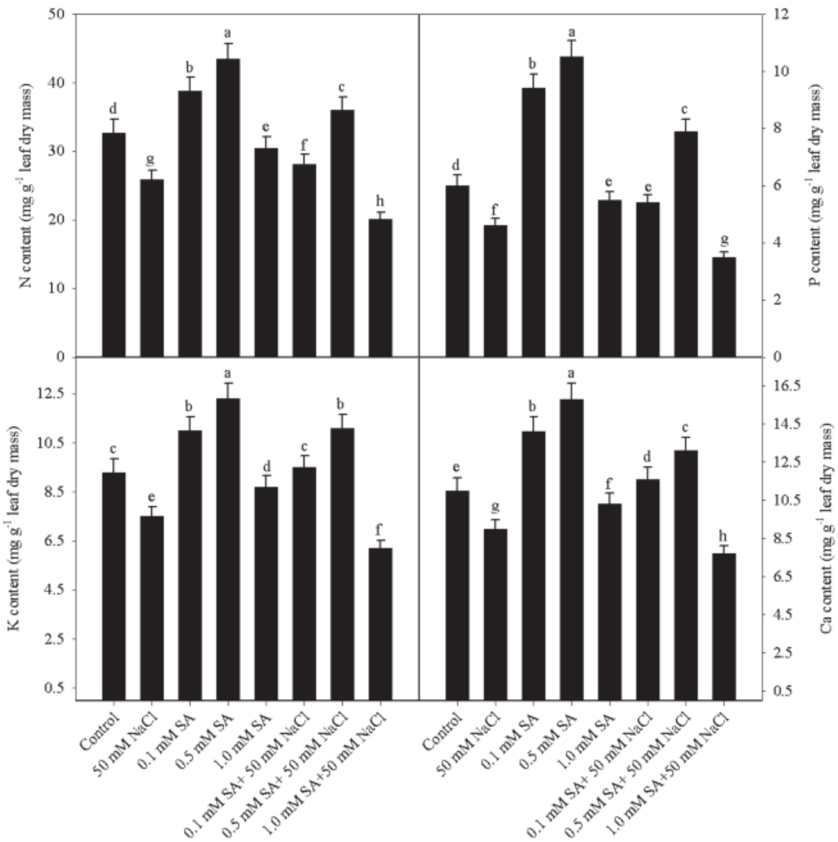

Figure 2. Effect of salicylic acid spray at 15 days after sowing (DAS) on N, P, K, and Ca content of mungbean (*Vigna radiata* L.) plants grown with 0 or 50 mM NaCl at 30 DAS. Values are mean±SE. Data labeled by the same letter did not differ significantly at P<0.05.

Content of H_2O_2 and thiobarbituric acid reactive substances, and electrolyte leakage

The content of H_2O_2 and TBARS, and electrolyte leakage increased several-fold under NaCl in comparison to the control (Figure 3). SA concentrations (0.1-1.0 mM) reduced H_2O_2 and TBARS content compared to the control, but did not differ significantly in effect under the nonsaline condition. However, electrolyte leakage was not significantly affected by SA application. SA applied at 0. 1 mM and 0. 5 mM on plants grown with 50 mM NaCl exhibited less content of H_2O_2 and TBARS, and electrolyte leakage than plants grown with 50 mM NaCl alone. The effect of 1.0 mM SA was similar to 0.5 mM SA in reducing H_2O_2 and TBARS content, and electrolyte leakage.

Antioxidants

The activity of antioxidant enzymes increased under salt stress. Salt stress increased SOD, GR, and APX activity by 30.0%, 8.7%, and 35.9%, respectively, compared to the control. Under the nonsaline condition, the application of 0.5 mM SA resulted in maximum increase in activity of SOD by 74.8%, GR by 34.8%, and APX by 95.3% compared to the control. Under the saline condition also, application of 0.5 mM SA on plants maximally increased the activity of antioxidant enzymes, showing about a two-fold increase in SOD and APX, while GR activity was increased to a lesser extent. The application of 1.0 mM SA on plants grown without or with 50 mM NaCl proved inhibitory and decreased the activity of antioxidant enzymes compared to the control (Figure 4).

Glutathione content in NaCl-grown plants increased twice in comparison to the control. Glutathione content increased about four times with the application of 0.5 mM SA in plants grown without NaCl (Figure 5). SA at 0.1 and 0.5 mM applied on plants grown with 50 mM NaCl further increased glutathione and the maximum increase of about five times was noted with 0.5 mM SA. The application of 1.0 mM SA resulted in glutathione content less than the control (Figure 5).

Photosynthetic traits

The activity of carbonic anhydrase, photosynthesis, stomatal conductance, intercellular CO_2 concentration, transpiration rate, and water use efficiency were decreased by 36.4%, 18.2%, 9.7%, 10.8%, 15.8%, and 20.5%, respectively, owing to 50 mM NaCl, compared to the control (Figure 6). The effect of SA on photosynthetic characteristics was positive under the nonsaline condition. The increases in carbonic anhydrase activity, photosynthesis, stomatal conductance, intercellular CO_2 concentration, transpiration rate, and water use efficiency with the application of 0.5 mM SA were 20.4%, 22.2%, 19.1%, 19.2%, 19.3%, and 22.3% in comparison to the water-sprayed control. The application of 0.5 mM SA resulted in alleviating the effects of salt stress, restoring carbonic anhydrase activity and photosynthesis to the level of the control, and water use efficiency higher than the control. In contrast, SA application reduced stomatal conductance, intercellular CO_2 concentration, and transpiration (Figure 6).

Yield traits

Plants grown with 50 mM NaCl exhibited a decrease of 13.3% in pod length, 19.1% in pod number, 21.8% in seed number, and 20.1% in seed yield in comparison to the control. The application of 0.5 mM SA grown under 0 mM NaCl (control) increased pod length by 19.9%, pod number by 19.9%, seed number by 20.2%, and seed yield by 20.1% in comparison to the control (Figure 7). SA at 0.5 mM concentration was found to alleviate the salt stress effects maximally, and nullified the effect of NaCl when compared with the control. SA at 1.0 mM was found to be inhibitory and increased the decrease in yield traits caused by 50 mM NaCl alone (Figure 7).

Discussion

The present study was undertaken to improve our understanding of the physiological mechanisms involved in salt tolerance and induction of such mechanisms by salicylic acid. Foliar application of SA had an ameliorative effect under both nonsaline and saline conditions. The application of 0.5 mM SA on NaCl grown plants substantially decreased the content of leaf Na^+, Cl^-, H_2O_2, and TBARS, and electrolyte leakage, and increased leaf N, P, K, and Ca content, and activity of antioxidant enzymes and glutathione. This treatment resulted in reduced negative effects of salt stress on growth, photosynthesis, and yield. The application of higher concentration of SA (1.0 mM) on plants grown with NaCl further enhanced the negative effects of NaCl on growth, photosynthesis, and yield and thus proved inhibitory.

Salt-induced reduction in growth and photosynthesis has been attributed to high Na^+ and Cl^-,[25] disturbance in the accumulation of nutrients, reduction in water potential and increase in osmotic potential, inhibition of photochemical processes,[8,43] and the increased production of ROS in the chloroplast.[44,45] The alleviation of salt stress effects on growth and photosynthesis with 0.5 mM SA was a result of increased content of leaf N, P, K, and Ca and decreased content of leaf Na^+ and Cl^-.

Gunes *et al.*[16] reported that the application of SA increased calcium, copper, magnesium, manganese, potassium, and zinc concentration in maize under salt stress. It may be suggested that 0.5 mM SA application resulted in high K^+ concentration and low concentration of Na^+ in the cytosol by regulating the expression and activity of K^+ and Na^+ transporters and H^+ pumps that generate the driving force for transport. Further, the accumulation of Ca^{2+} in plants receiving SA possibly maintained membrane integrity and helped in reducing the toxic effects of Na^+ and Cl^- ions. Ca^{2+} is considered as an obligate intracellular

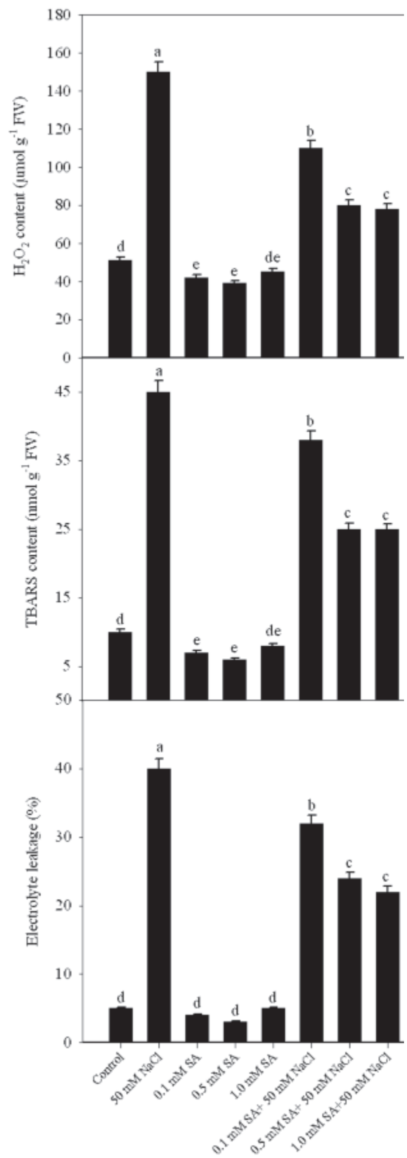

Figure 3. Effect of salicylic acid spray at 15 days after sowing (DAS) on H_2O_2 content, TBARS content, and electrolyte leakage of mungbean (*Vigna radiata* L.) plants grown with 0 or 50 mM NaCl at 30 DAS. Values are mean±SE. Data labeled by the same letter did not differ significantly at P<0.05.

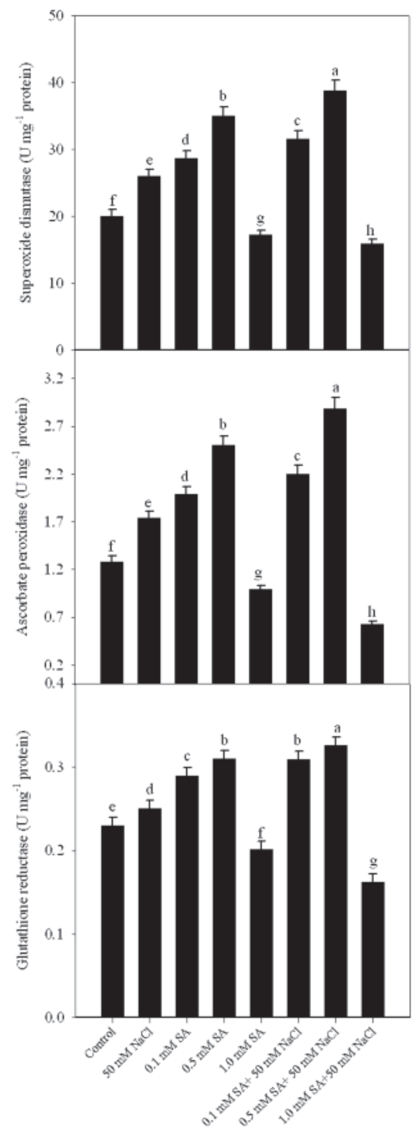

Figure 4. Effect of salicylic acid spray at 15 days after sowing (DAS) on superoxide dismutase, ascorbate peroxidase, and glutathione reductase of mungbean (*Vigna radiata* L.) plants grown with 0 or 50 mM NaCl at 30 DAS. Values are mean±SE. Data labeled by the same letter did not differ significantly at P<0.05.

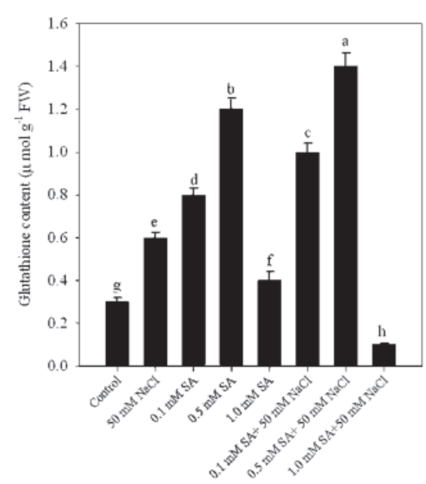

Figure 5. Effect of salicylic acid spray at 15 days after sowing (DAS) on the content of glutathione (GSH) in the leaves of mungbean (*Vigna radiata* L.) plants grown with 0 or 50 mM NaCl at 30 DAS. Values are mean±SE. Data labeled by the same letter did not differ significantly at P<0.05.

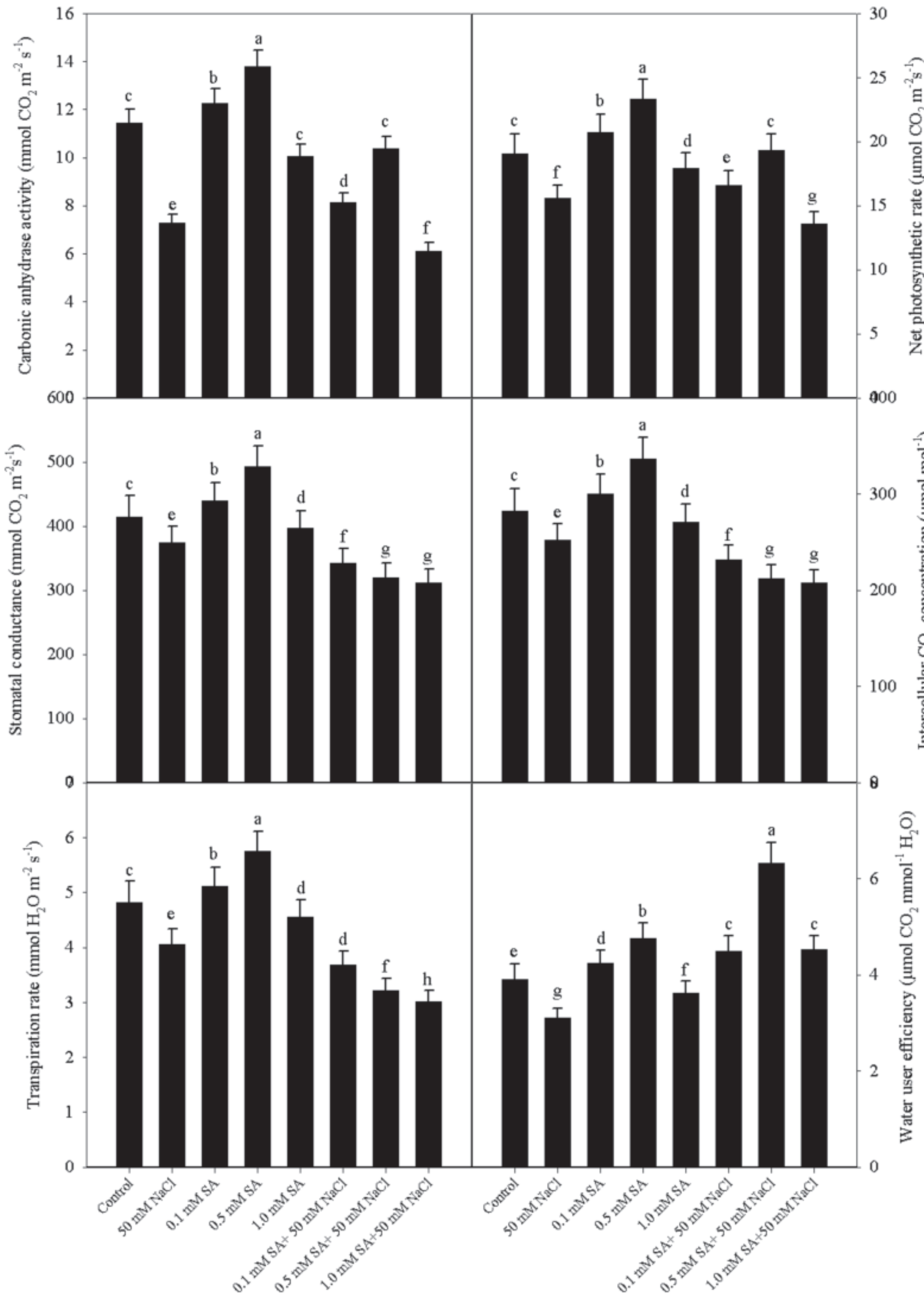

Figure 6. Effect of salicylic acid spray at 15 days after sowing (DAS) on carbonic anhydrase activity, photosynthesis, stomatal conductance, intercellular CO^2 concentration, transpiration rate, and water use efficiency of mungbean (*Vigna radiata* L.) plants grown with 0 or 50 mM NaCl at 30 DAS. Values are mean±SE. Data labeled by the same letter did not differ significantly at $p<0.05$.

messenger coordinating responses to numerous developmental cues and the environment.[46] An interaction of SA and Ca^{2+} in signaling has been shown in tobacco cell suspension culture.[47] Calcium has been shown to maintain K^+/Na^+ ion selectivity[48] and be involved in defense mechanisms induced by stress.[49] Externally supplied Ca^{2+} has been shown to reduce toxic effects of NaCl by facilitating high K^+/Na^+ selectivity.[50] The protective role of SA in membrane integrity and regulation of ion uptake has been reported earlier.[21-25,51,52] Experimental evidence also implies that the increase of Ca^{2+} uptake is associated with the rise of ABA under salt stress and thus contributed to membrane integrity maintenance, which enables the plant to regulate uptake and transport under salt stress.[47]

The salt-induced reduction in photosynthesis was reversed by 0.5 mM SA application. This reversal is the mitigation of NaCl effects on CA activity resulting in promotion of water-use efficiency under salt stress. The reduction in stomatal conductance, transpiration rate, and intercellular CO_2 concentration under salt stress can be related to the findings of Larque-Saavedra,[53] who observed that the exogenous application of SA had an antitranspiration effect on the leaves of *Phaseolus vulgari*s and caused reduction in stomatal conductance resulting in a decrease in intercellular CO_2 concentration. The alleviation of salt-induced effects on photosynthesis by 0.5 mM SA with the decrease in stomatal conductance and intercellular CO_2 concentration suggests that the effect of 0.5 mM SA on photosynthesis

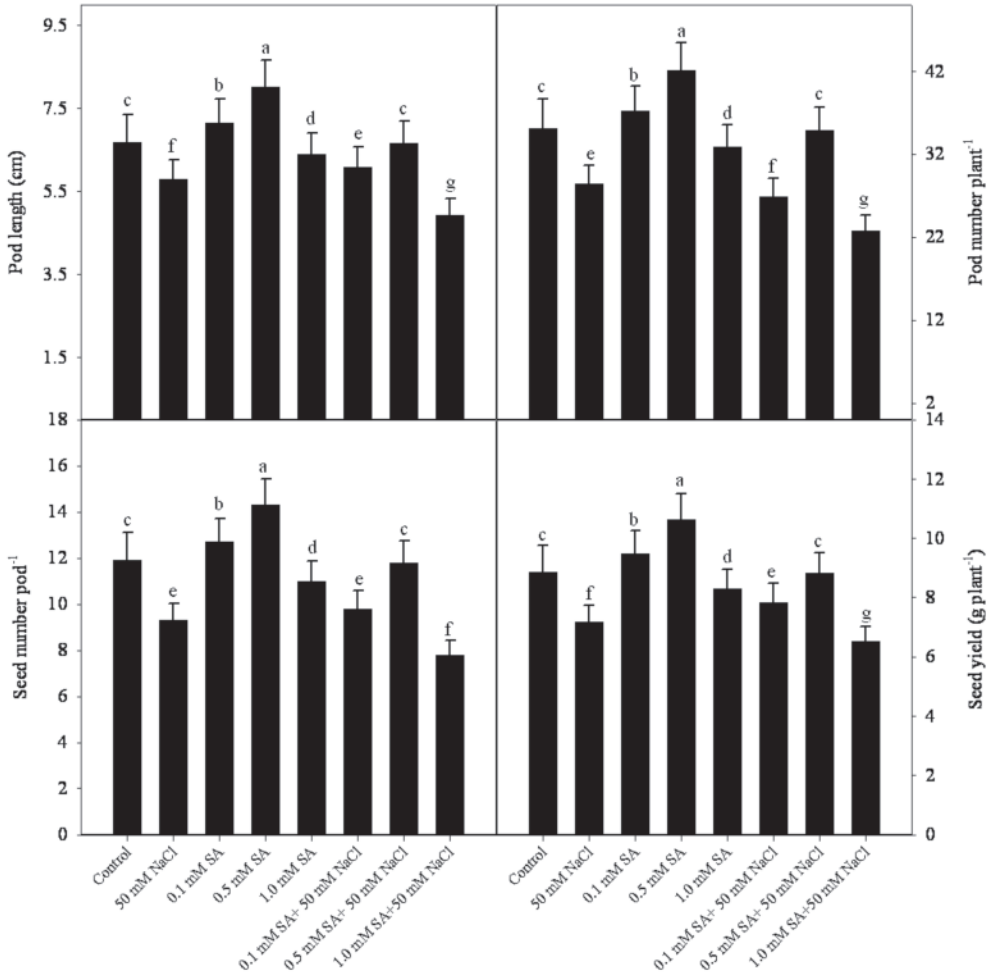

Figure 7. Effect of salicylic acid spray at 15 days after sowing (DAS) on pod length, pod number, seed number, and seed yield of mungbean (*Vigna radiata L.*) plants grown with 0 or 50 mM NaCl at 30 DAS. Values are mean±SE. Data labeled by the same letter did not differ significantly at P<0.05.

under salt stress probably is a result of other factors. This may be attributed to the increased allocation of N to the leaf with 0.5 mM SA application under salt stress increasing the content and activity of ribulose 1,5 bisphosphate carboxylase.[23-24] Furthermore, higher photosynthesis under decreased intercellular CO_2 concentration suggests that CO_2 was utilized more efficiently with 0.5 mM SA application under the salt stress condition. This is shown also with the concurrent changes in CA activity. CA is an enzyme responsible for the reversible hydration of CO_2 and has shown a strong relationship with mustard photosynthesis.[54] The effect of SA on CA activity under salt stress has not been studied earlier. Rai *et al.*[55] have reported the reversal of ABA-induced stomatal closure by SA application. In contrast, de Bruxelles *et al*.[56] suggested that changes in ABA were responsible for the alteration of salt stress genes. ABA alleviated the inhibitory effect of NaCl on photosynthesis, growth, and translocation of assimilates[57] through stomatal closure by altering ion fluxes in guard cells under salt stress.[58]

Salt stress induces water deficit and increases ionic and osmotic effects leading to oxidative stress and formation of ROS.[59] In the present study, maximum oxidative stress as noted in terms of content of H_2O_2 and TBARS, and electrolyte leakage was observed under salt stress, which was alleviated to some extent with the application of 0.5 mM SA. Plants initiate several mechanisms including the antioxidant system for protection against stress.[3] In the present investigation, maximum activity of antioxidant enzymes SOD, APX, and GR was with NaCl and 0.5 mM SA application. The increases in the activity of antioxidant enzymes following SA application could be the indicator of build-up of a protective mechanism to reduce oxidative damage induced by salt stress. Efficient destruction of H_2O_2 in the chloroplast requires the induction of APX and activation of CO_2-fixing enzymes.[60] A major fraction of the total leaf GR activity is associated with the chloroplast.[61] The role of GR and glutathione in H_2O_2 scavenging in plant cells has been well established.[62] In addition to catalyzing the rate-limiting last step of the ascorbate-glutathione cycle, it is involved in maintaining a high ratio of reduced glutathione to oxidized glutathione. Glutathione, a non-protein thiol, is an essential component of the cellular antioxidant defense system keeping ROS under control.[63] A substantial increase in glutathione content with 0.5 mM SA application under salt stress helped in reversing the effects of salt-induced ROS on growth and photosynthesis. The increase in the activity of antioxidant enzymes with SA has been reported under different stress conditions.[64] Recently, it has been shown that overexpression of the soybean *GmERF3*, an AP2/ERF-type transcription factor, increased tolerance to salt, drought, and disease in transgenic tobacco.[65]

Yield is the final manifestation of the growth and photosynthetic processes. Salt-induced reduction in yield was alleviated by the application of 0.5 mM SA through its effect on the contents of ions and nutrients and activity of antioxidant enzymes and glutathione content. Experiments conducted on wheat by Arfan *et al.*[24] and on maize by Gunes *et al*.[25] have reported the ameliorative effect of SA on yield.

The application of higher concentration of SA (1.0 mM) proved inhibitory on the characteristics studied under nonsaline and saline conditions. Durner and Klessig[66] provided the evidence that inhibition of APX with the higher concentration of SA blocks the H_2O_2 degrading pathway in the plant cell leading to increased levels of endogenous H_2O_2. Treatment of tobacco plants with 1.0 mM SA has been found to induce a 50-60% increase in endogenous H_2O_2 levels. The inhibitory effect of a high concentration of exogenously applied SA on maize growth was reported by Nemeth *et al.*[29] Borsani *et al*.[30] demonstrated that transgenic *Arabidopsis* plants with lower endogenous SA were better able to resist the oxidative damage caused by salt stress than were the wild type plants. The application of 1.0 mM SA under salt stress resulted in inhibition in growth, photosynthesis, and yield. Amin *et al.*[67] have reported inhibition in the wheat yield with higher SA concentration.

We concluded that SA alleviates the negative effect of salt stress on photosynthesis depending on the concentration of SA used. Maximum alleviation was found with 0.5 mM SA application while 1.0 mM SA proved inhibitory. The application of 0.5 mM SA alleviated salt stress effects by increasing nutrients, content and antioxidant metabolism.

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