RESEARCH PAPER

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Role of salicylic acid in induction of plant defense system in chickpea (*Cicer arietinum* L.)

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Key words: induced resistance, peroxidase, phytohormones, polyphenol oxidase

Abbreviations: ANOVA, analysis of variance; EDTA, ethylenediamine tetra acetic acid; FW, fresh weight; GAE, gallic acid equivalents; H_2O_2, hydrogen peroxide; IUg^{-1} FW, international units per gram fresh weight; m h, million hectares; m t, million tons; KI, potassium iodide; Na_2CO_3, sodium carbonate; PAL, phenylalanine ammonia lyase; POD, peroxidase; PPO, polyphenol oxidase; PVP, polyvinyl pyrolidone, ROS, reactive oxygen species; SOD, superoxide dismutase; TCA, trichloroacetic acid; Tris-HCl, tris (hydroxymethyl) amino methane hydrochloride

Salicylic acid (SA), a plant hormone plays an important role in induction of plant defense against a variety of biotic and abiotic stresses through morphological, physiological and biochemical mechanisms. A series of experiments were performed to evaluate the biochemical response of the chickpea (*Cicer arietinum* L.) plants to a range of SA concentrations (1, 1.5 and 2 mM). Water treated plants were maintained as control. Activities of peroxidase (POD) and polyphenol oxidase (PPO) were evaluated and amounts of total phenols, hydrogen peroxide (H_2O_2), and proteins were calculated after 96 h of treatment. Plants responded very quickly to SA at 1.5 mM and showed higher induction of POD and PPO activities, besides the higher accumulation of phenols, H_2O_2 and proteins. Plants treated with SA at 2 mM showed phytotoxic symptoms. These results suggest that SA at 1.5 mM is safe to these plants and could be utilized for the induction of plant defense.

Introduction

Plants face innumerable challenges ranging from environmental stresses such as drought, flood, temperature fluctuation, and microbial to insect attack. The oxidative state of plants has been found to play an important role against many biotic and abiotic stresses.[1-3] Plant defense against these stresses is mediated through various signaling pathways that lead to the production of many defensive proteins and non-protein compounds.[2,4] Plant phytohormones such as abscisic acid, jasmonic acid, ethylene and salicylic acid (SA) are important components of different signaling pathways involved in plant defense.[2-6] SA mediates the phenylpropanoid pathway, while as JA mediates the octadecanoid pathway.[5,6] The former plays an important role against pathogens and some insect pests and abiotic stresses, while as the latter is mostly meant for the defense against insect pests and some pathogens.[2,4,7,8] Exogenous application of SA and JA manipulates various physiological, biochemical and molecular processes in plants including antioxidative enzyme activities.[2-10] Moreover, SA regulates the components of its own signaling pathway besides getting involved in cross-talk with other pathways mediating plant resistance. It has been proposed that SA affects the plant growth under stress through nutrient uptake, water relations, stomatal regulation and photosynthesis.[9] It regulates the activities of various enzymes such as, peroxidase (POD), polyphenol oxidase (PPO), superoxide dismutase (SOD), phenylalanine ammonia lyase (PAL) etc., which are the major components of induced plant defense against biotic and abiotic stresses.[2,3,8-10] PODs constitute an important group of defense enzymes that defend plants against various stresses.[2,3,8,11] PPOs also play a pivotal role in plant defense.[2,3,12,13] Plant phenolic compounds are the most abundant and important group of defensive compounds that mediate plant defense.[2,13,14] Oxidative burst i.e., the generation of reactive oxygen species (ROS) is the immediate response of plants to elicitor treatment including SA. ROS mediate different signaling pathways to regulate the expression of genes associated with plant defense mechanisms,[15] and their role as second messengers in hormone signaling pathways has been well documented.[1,16]

Chickpea (*Cicer arietinum* L.) is an important legume crop in the semi-arid tropics of the world. India is the world leader in chickpea production. In India, chickpea is cultivated on 8.68 mha with annual production of 8 mt and an average yield of 844 $kgha^{-1}$.[17] However, it is under heavy stresses due to various biotic and abiotic factors that results in yield losses. The present study was performed to investigate the response of chickpea plants to exogenous application of SA and to examine the effect of SA on the activities of antioxidant enzymes (POD and PPO), amount of total phenols, hydrogen peroxide and total proteins in this crop, since these are the most studied components implicated in plant defense against biotic and abiotic stresses.

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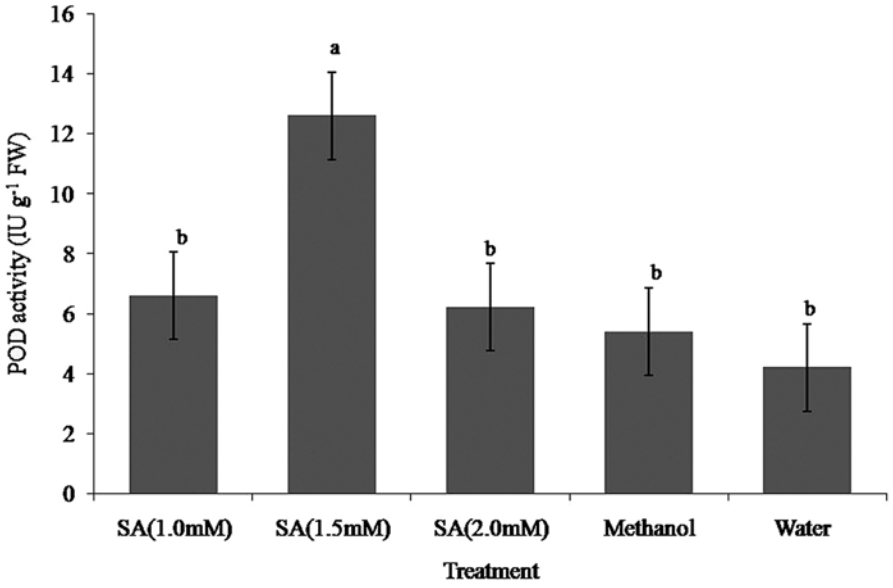

Figure 1. Peroxidase activity (IUg^{-1} FW) of chickpea plants at 96 h after treatment with SA. Bars (Mean ± SEM) with same letter(s) are not significantly different by Tukey's test ($p \leq 0.05$); n = 10 for each treatment.

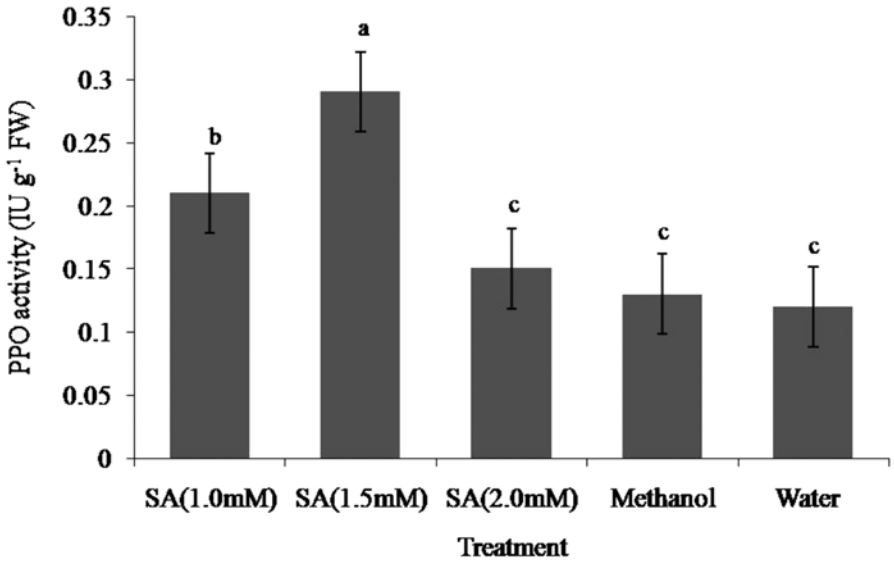

Figure 2. Polyphenol oxidase activity (IUg^{-1} FW) of chickpea plants at 96 h after treatment with SA. Bars (Mean ± SEM) with same letter(s) are not significantly different by Tukey's test ($p \leq 0.05$); n = 10 for each treatment.

Results

Enzyme activities. Peroxidase activity was significantly higher in the plants sprayed with SA (1.5 mM) ($F_{4,19} = 67.8$, $p < 0.01$) as compared with the plants treated with SA 1.0 and 2.0 mM, methanol and water sprayed control plants (**Fig. 1**). No significant difference was observed in POD activity in plants sprayed with SA 1.0 and 2.0 mM, methanol and water ($p > 0.05$). Significant elevation of the PPO activity was observed in plants sprayed with SA (1.5 mM) ($F_{4,19} = 54.8$, $p < 0.01$) followed by the plants sprayed with SA 1.0 mM (**Fig. 2**). Differences were not significant among the plants sprayed with SA (2.0 mM), methanol and water.

Phenolic content. Treatment with SA elevated the total phenolic content. However, significantly higher contents were shown by the plants sprayed with SA at 1.5 mM ($F_{4,19} = 33.2$, $p < 0.01$) (**Fig. 3**). No significant differences were recorded in the phenolic contents of plants sprayed with SA at 1.0 and 2.0 mM. Least amount of phenols was observed in plants sprayed with methanol and water.

Hydrogen peroxide content. Hydrogen peroxide content was higher in plants sprayed with SA at 1.5 and 2.0 mM ($F_{4,19} = 37.8$, $p < 0.05$), followed by those sprayed with SA 1.0 mM (**Fig. 4**). No significant difference was shown by methanol and water sprayed plants.

Protein content. There was an elevation in protein content in all the SA treatments, however; significantly higher protein content was recorded in plants sprayed with SA at 1.5 and 2.0 mM concentration, followed by the plants sprayed with SA at 1.0 mM concentration (**Fig. 5**). No significant differences were observed between plants sprayed with methanol and water.

Discussion

Pre-treatment of plants with different biotic (pathogens and insect pests) and abiotic inducers (chemicals) induce plant resistance that defends the plants against their subsequent attack. The plant phytohormones induce plant defense against many biotic and abiotic stresses.[1-11] This induction of plant defense is mediated through various physiological, biochemical and molecular mechanisms.[1-10] Salicylic acid is an important and well-studied endogenous plant growth regulator that generates a wide range of metabolic and physiological responses in plants involved in plant defense in addition to their impact on plant growth and development.[4,6-10] SA also activates the generation of ROS and other defensive processes such as hypersensitive response and cell death.[9] Biochemical basis of induced defense has been found to be very active and dynamic with profound effect on the stress causing agents, thereby enabling the plants to withstand them.[14]

A greater elevation in POD activity was found in plants treated with SA at 1.5 mM than the plants treated with SA at 1 and 2 mM. At 2 mM, phytotoxicity was observed in plants that could have led to the reduction in POD activity. Most of the leaves in the plants treated with 2 mM SA turned yellowish and showed wilting (data not shown). Similar results were observed for PPO, where SA at 1.5 mM induced significantly higher activity followed by SA at 1 mM. Least activity was observed in plants treated with methanol and water. Since no significant differences were recorded in POD and PPO activities between methanol and water treated plants, it shows that methanol used for preparation of SA solution has no role in induction of the activities of these enzymes. Low activity of POD and PPO in plants treated with SA at 2 mM could be due to the phytotoxicity of plant at higher concentration.[18] Moreover, greater induction of POD and PPO activities by SA at 1.5 mM depicts that SA at this concentration is safe and can be utilized in induction of plant resistance. POD and PPO are the important enzymes involved in plant defense against many biotic and abiotic stresses.[2,3,19] Elicitors such as jasmonic acid, SA and ethylene have been found to induce such enzymes in plants.[2-11] Induction of these enzymes by exogenous application of SA and their role in plant defense against various stresses including salinity,[10] drought,[21] pathogens,[4,22] and insects,[8,20,23] has been studied in many plants.

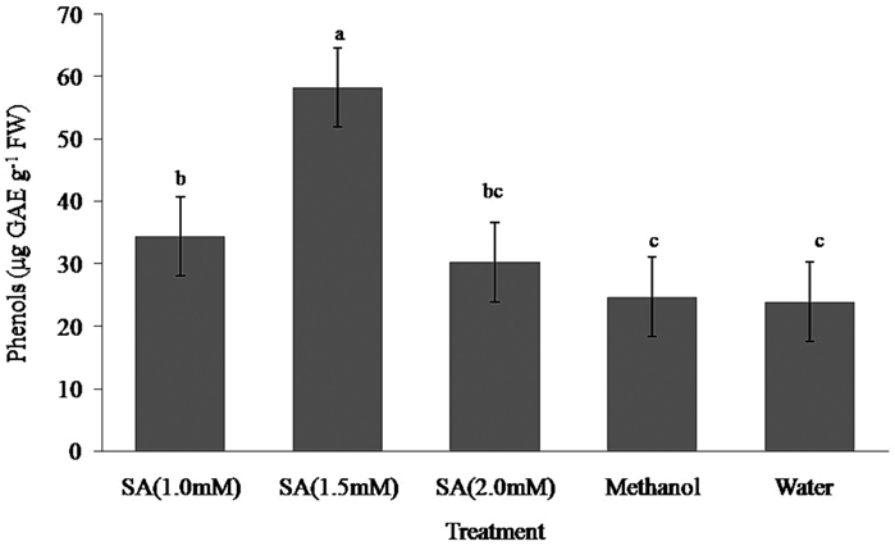

Figure 3. Total phenols (µg $GAEg^{-1}$ FW) of chickpea plants at 96 h after treatment with SA. Bars (Mean ± SEM) with same letter(s) are not significantly different by Tukey's test ($p \leq 0.05$); GAE = Gallic acid equivalents; n = 10 for each treatment.

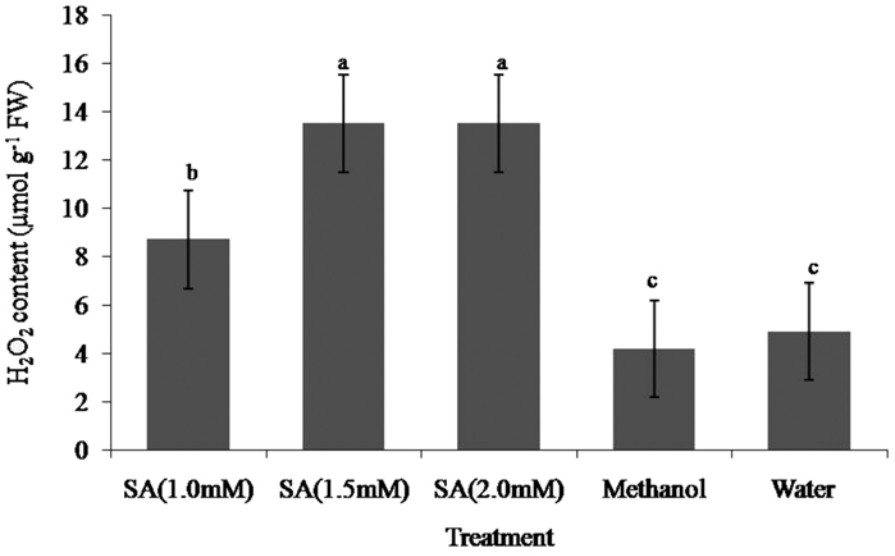

Figure 4. Hydrogen peroxide content (µmolg[1] FW) of chickpea plants at 96 h after treatment with SA. Bars (Mean ± SEM) with same letter(s) are not significantly different by Tukey's test ($p \leq 0.05$); n = 10 for each treatment.

Phenolic content was induced in plants on treatment with SA. Higher induction was observed in plants treated with SA at 1.5 mM than the other treatments. Phenolic content of the plants treated with SA 1 mM and 2 mM were not significantly different. Phytotoxicity of plants at higher concentration of SA may have led to low phenolic content.[18] Phenolic compounds defend plants against a number of biotic and abiotic stresses.[2,3,14,19] Oxidation of phenols produces many defensive compounds that alter the plant physiology and metabolism, which in turn enable it to withstand various stresses either directly or by mediating different plant signaling pathways.[19] Furthermore, ROS such as superoxide anion, hydroxide radicals, H_2O_2 and singlet oxygen produced by oxidation of phenols activate plant defense enzymes.[1,15]

Hydrogen peroxide is an important signaling molecule that mediates the synthesis of many defensive compounds in plants in response to biotic and abiotic stresses.[1,11,15,24] Hydrogen peroxide content was elevated in the SA-treated plants. However, plants treated with SA at 1.5 mM had higher H_2O_2 than other treatments. Production of ROS in plants in response to stress is a common phenomenon.[1-3,15,24] They play a potent role in plant defense against biotic and abiotic stresses either by direct toxicity or by activating defensive enzymes.[1,15] Among ROS, H_2O_2 is very important, because it is stable and easily diffusible through the cell membranes.[1] H_2O_2 triggers several physiological and molecular processes in plants that signal the production of

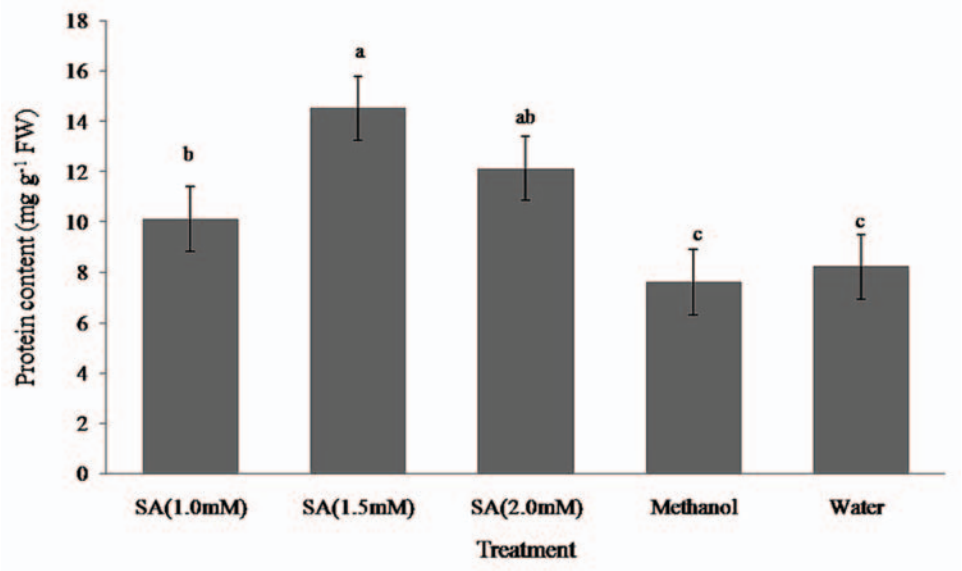

Figure 5. Protein content (mgg^{-1} FW) of chickpea plants at 96 h after treatment with SA. Bars (Mean ± SEM) with same letter(s) are not significantly different by Tukey's test ($p \leq 0.05$); n = 10 for each treatment.

various defensive compounds and enzymes, which in turn modify plant resistance against stresses.[1,15,24,25] It has been suggested that SA application leads to the uptake of exogenous SA into the veins that result in H_2O_2 accumulation.[26] Furthermore, H_2O_2 has been reported to increase the secondary metabolite production in plants.[1,27] Our results are in accordance with the earlier reports where exogenous application of SA induced H_2O_2 content in plants.[26,28] In addition, the ROS produced on SA treatment mediates the accumulation of cytosolic calcium that in turn triggers additional physiological processes.[29]

Proteins play an important role in plant defense in the form of various defense enzymes and other protein based non enzymatic compounds.[2,3,13,30] Plants treated with SA 1.5 mM exhibited higher protein content followed by SA 2 mM and 1 mM (**Fig. 5**). The higher protein content could be due to the synthesis of defensive enzymes and other protein based compounds by plants after treatment with SA. Least protein content was found in control plants. Defense related enzymes and other protein based defensive compounds have been reported to get elevated in plants in response to various stresses.[2,3,9-11,13,30] There are a number of reports where role of proteins in induced resistance of plants has been well documented.[2-4,9-11,13,30]

Methods and Materials

Chemicals. The chemicals used in this study were of analytical grade. 2-mercaptoethanol (RM2895), Tris-hydrochloride (RM613), Polyvinylpyrolidone (PVP: RM854), EDTA (RM1279), Disodium hydrogen phosphate (RM1154), Sodium dihydrogen phosphate (RM1155), Guaiacol (RM1118), Gallic acid (RM233), Salicylic acid (RM1476) was obtained from HiMedia. Pyrocatechol (120-80-9) was obtained from Central Drug House. Coomassie brilliant blue-G250 (0240109, Sisco Research Lab). Bovine serum albumin (BSA: 54155), potassium iodide (KI: 39631) and Sodium carbonate (Na_2CO_3: 20240) were obtained from S.d. Fine Chemicals Ltd., Folin-Ciocalteau reagent (19058) and Trichloroacetic acid (TCA: 10286) were obtained from Merck.

Chickpea plants (*C. arietinum* L.). Seeds of the chickpea (Kabuli) were obtained from Saravana Stores, T. Nagar, Chennai, Tamil Nadu. Chick pea seeds were sown in plastic pots (30 cm diameter). The pots were filled with a potting mixture of soil and vermicompost (2:1). The plants were watered as needed. The pots were maintained in cages to avoid any stress due to insects or other agents. Twenty day old chickpea plants were used for the study. The experiment was repeated thrice.

Salicylic acid application. Plants were grouped into five sets each with ten replicates. Set I: Sprayed with SA (1 mM); Set II: Sprayed with SA (1.5 mM); Set III; sprayed with SA (2 mM); Set IV; sprayed with methanol; Set V: sprayed with water. After 4 d of treatment, leaves were collected from the plants for evaluation of different biochemical attributes.

Enzyme assays. Newly emerged fully expanded leaves were collected from treated plants and immediately frozen in liquid nitrogen. Leaves (0.5 g) were homogenized in 3 ml of ice cold 0.1 M TRIS-HCl buffer (pH 7.5) containing 2-mercaptoethanol (5 mM), 1% polyvinylpyrolidone (PVP) and 0.5 mM EDTA. The homogenate was centrifuged at 16,000x g for 25 min and the supernatant was used as enzyme source. All spectrophotometric analyses were performed on HITACHI UV-2010 spectrophotometer. Peroxidase activity was determined as per the method of Shannon et al. with slight modifications. To the reaction mixture (2.9 ml) containing 0.1 M sodium phosphate buffer (pH 6.5), 0.8 mM H_2O_2 and 5 mM Guaiacol, 0.1 ml of enzyme source was added. Absorbance was read at 470 nm for 2 min at 15 sec intervals. Enzyme activity was measured as IUg^{-1} FW (International Units g^{-1} FW). One unit of POD activity was defined as the change in absorbance by 0.1 units per minute under conditions of assay. Polyphenol oxidase activity was estimated as per the method of Mayer and Harel[32] with slight modifications. To the reaction mixture (2.9 ml of 0.1 M sodium phosphate buffer, pH 6.8), 0.1 ml of enzyme source and 0.1 ml of substrate (0.05 M catechol) were added. Absorbance was read at 420 nm for 3 min at 30 sec interval. Enzyme activity was measured as IUg^{-1} FW. One unit of PPO was defined as the change in absorbance by 0.1 units per minute under conditions of assay.

Phenolic content. Phenolic content of treated leaves was evaluated using a slightly modified version of Folin-Ciocalteau

assay as described by Zieslin and Ben-Zaken.[33] Fresh leaves (0.5 g) were extracted in 3 ml of 80% methanol and agitated for 15 min at 70°C. Briefly, 0.1 ml of methanol extract was added to 2 ml of 2% Na_2CO_3. After incubation for 5 min, 0.1 ml of Folin-Ciocalteau reagent was added and the solution was incubated for 10 min at room temperature. The absorbance of blue color was measured at 760 nm. Gallic acid was used as a standard and a calibration curve was prepared with a range of concentrations. Phenolic content was expressed as μg Gallic acid equivalents g^{-1} FW (μg GAEg^{-1} FW).

Hydrogen peroxide. For the estimation of H_2O_2, method of Noreen and Ashraf[11] was followed. Fresh leaf tissue (0.1 g) was homogenized in 2 ml of 0.1% (w/v) TCA in a pre-chilled pestle and mortar. The homogenate was centrifuged at 12,000x g for 15 min and the supernatant was collected. Absorbance of the reaction mixture consisting of 0.5 ml supernatant, 0.5 ml sodium phosphate buffer (pH 7.0) and 1 ml of 1 M KI was read at 390 nm. The H_2O_2 content was determined by using an extinction coefficient of 0.28 μMcm^1 and expressed as $\mu molg^{-1}$ FW.

Protein content. Protein was determined according to the method of Bradford[34] with minor modifications, using bovine serum albumin as a standard.

Statistical analysis. The replication data were pooled together and mean and standard error were calculated. All data were analyzed by analysis of variance (ANOVA) using SPSS (Version 15.1). When the treatment effects were statistically significant ($p \leq 0.05$), the Tukey's test was used to separate the means.

Conclusion

Salicylic acid at 1.5 mM was found to induce higher activities of POD, PPO and amounts of total phenols, H_2O_2 and protein content in chickpea plants as compared with the other treatments. Since the biochemical mechanism of induced resistance in plants is almost similar against biotic (insect pests, pathogens) and abiotic stresses, the present study could have the utility against different stresses. The results suggest that SA at this 1.5 mM concentration could be utilized for the induction of plant defensive system that will enable the plant to withstand many biotic and abiotic stresses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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