

Salicylic acid mediated changes in growth, photosynthesis, nitrogen metabolism and antioxidant defense system in *Cicer arietinum* L.

Q. Hayat¹, S. Hayat^{1,2}, M.N. Alyemeni², A. Ahmad¹

¹Plant Physiology Section, Department of Botany, Aligarh Muslim University, Aligarh, India

²Department of Botany and Microbiology, King Saud University, Riyadh, Saudi Arabia

ABSTRACT

The present study reveals that the foliar application of salicylic acid (SA), irrespective of the concentration used, generated an increase of dry mass per plant, nodule dry mass and leghemoglobin content in chickpea plants. The activity of nitrogenase (E.C 1.18.6.1), nitrate reductase (NR) (E.C. 1.6.6.1), glutamine synthetase (GS) (E.C 6.3.1.2), glutamate synthase (GOGAT) (E.C 1.4.7.1) and glutamate dehydrogenase (GDH) (E.C 1.4.1.3) increased as well. Among the three concentrations of SA, the order of response was found to be 10^{-5} mol/L > 10^{-6} mol/L > 10^{-4} mol/L > control.

Keywords: chickpea; glutamine synthase; leghemoglobin; nitrate reductase; nitrogenase

Salicylic acid (SA) is considered to be a potent plant hormone because of its diverse regulatory roles in plant metabolism (Hayat et al. 2010). SA is an endogenous plant growth regulator of phenolic nature that possesses an aromatic ring with a hydroxyl group or its functional derivative. SA is known to affect growth and bioproductivity in the crops (Hayat et al. 2010). It is also recognized that SA potentially generates a wide array of metabolic responses in plants and also affects photosynthetic parameters and plant water relations (Fariduddin et al. 2003, Hayat et al. 2005). The oxidative damage caused to the plants due to the increased generation of reactive oxygen species under stressful environment could be mitigated by the external application of SA (Hayat et al. 2010). A large body of literature demonstrates the effect of SA seed soaking on plant growth and development. However, very little is known about the effect of SA applied to foliage on plant growth and development. Keeping in view the diverse physiological roles played by SA, the present research was undertaken to improve our understanding of the effect of the various concentrations of SA applied as foliar spray on the growth, photosynthesis, nitrogen fixation and its assimilation in chickpea and to screen for the most effective concentration of SA.

MATERIAL AND METHODS

Plant material and growth condition. The certified seeds of chickpea (*Cicer arietinum* L.) cv. Avarodhi were purchased from the National Seed Corporation Ltd., New Delhi, India. The seeds were surface sterilized with 0.01% mercuric chloride solution followed by inoculation with *Rhizobium* and were sown in earthen pots (0.254 m in diameter) filled with sandy loam soil and farmyard manure (6:1) arranged under a simple randomized block design in the net house of the Botany Department of Aligarh Muslim University, India during the winter season (November–February). The temperature was 15–25°C and irrigation was done on alternate days during the experiment. At the stage of 30 days after sowing (DAS), the foliage of the plants was sprayed uniformly either with double distilled water (control), ethanol (5%), Tween-20 (0.5%) or with different concentrations (10^{-4} , 10^{-5} or 10^{-6} mol/L) of SA dissolved in ethanol to elucidate the effect of exogenous SA on plants. The plants were sampled at 90 DAS to assess various growth and physiological parameters. Tween-20 was used as a surfactant. Both ethanol and Tween 20 were sprayed separately to see whether the effects are solely by SA or by the combination of these.

Plant growth analysis. The plants were uprooted and washed under running tap water. The plants were then dried in an oven at 80°C for 72 h and then weighed to obtain their dry mass (DM). The nodules from each plant were picked and counted to note the number of nodules per plant. These nodules were then transferred to Petri dishes for overnight drying in an oven at 80°C. The dried materials were weighed to obtain DM of nodules per plant.

Biochemical analysis. The contents of leghemoglobin, carbohydrate and the activities of nitrogenase, glutamine synthetase (GS), glutamate synthase (GOGAT), glutamate dehydrogenase (GDH), and nitrate reductase (NR) in fresh leaf samples were measured as described in our earlier studies (Hayat et al. 2009). The leaf nitrogen content was estimated by employing the method of Lindner (1944) and the nitrate content in roots was estimated following the method of Singh (1988). The activities of carbonic anhydrase (CA), antioxidant enzymes and proline content were analyzed as described earlier in our studies (Hayat et al. 2008). The stomatal conductance (g_s) and net photosynthetic rate (P_n) in intact leaves were measured by LI-6400 portable photosynthesis system (LI-COR, Lincoln, USA), between 11:00 and 12:00 h.

Statistical analysis. Each observation was replicated three times. The treatment means were compared by analysis of variance (ANOVA) using the SPSS software version 10 (SPSS, Chicago, USA). Least significant difference (LSD) was calculated at 5% level of probability.

RESULTS

Growth and nodulations. The exogenous application of SA resulted in an increase of all growth

characteristics. Maximum response was generated by the foliar application of 10^{-5} mol/L of SA, where DM per plant was increased by 31.2%, nodule number by 54.3% and their DM by 59.0% over that of the control. Foliar spray of plants with 5% ethanol or 0.5% Tween-20 had no significant effect on all the growth parameters and revealed the values almost comparable with that of control (Table 1).

Leghemoglobin and carbohydrate content in nodules. Among the three concentrations (10^{-4} , 10^{-5} or 10^{-6} mol/L) of SA, 10^{-5} mol/L proved to be the best and significantly increased the values of leghemoglobin and carbohydrate content by 39.5% and 20.6% respectively, over that of the control. On the other hand, treatment of plants with ethanol or Tween-20, revealed the values that did not differ significantly from that of control (Table 1).

Nitrogenase, GS, GOGAT and GDH activities in nodules. Treatment of the plants with SA, irrespective of the concentration, resulted in a significantly higher activity of these enzymes, at both the sampling stages, compared to the control. However, among the three concentrations of SA, 10^{-5} mol/L proved to be the best and significantly enhanced the activities of nitrogenase (18.7%), GS (19.7%), GOGAT (47.5%) and GDH (59.6%), respectively, compared to the control. On the other hand, spraying of plants with ethanol or Tween-20, had no significant effect on the activities of these enzymes (Figures 1a–d).

NR activity in leaves. The foliar applied SA increased the activity of NR, where maximum response was generated by a concentration of 10^{-5} mol/L and was 32.1% higher than that of the control. The order of response was found to be 10^{-5} mol/L > 10^{-6} mol/L > 10^{-4} mol/L > control (Figure 1e).

Table 1. Effect of ethanol, Tween-20 and different concentrations of salicylic acid (SA) (10^{-4} , 10^{-5} or 10^{-6} mol/L) on dry mass per plant (g), nodule number, nodule dry mass (mg), nodule leghemoglobin content (mmol/g FM) and carbohydrate content (%) in *Cicer arietinum* at 90 DAS. Data are the mean of three independent replicates (\pm SE)

Treatments	Dry mass per plant	Nodule number	Nodule dry mass	Leghemoglobin content	Carbohydrate content
Control (DDW)	3.2 \pm 0.04	48.0 \pm 2.1	39 \pm 0.6	43 \pm 1.0	18.85 \pm 0.20
Ethanol (5%)	3.1 \pm 0.07	48.0 \pm 1.5	40 \pm 1.1	44 \pm 0.6	18.63 \pm 0.20
Tween-20 (0.5%)	3.1 \pm 0.08	47.3 \pm 0.8	39 \pm 0.5	43 \pm 0.6	18.93 \pm 0.30
SA (10^{-4} mol/L)	3.5 \pm 0.04	49.6 \pm 1.4	45 \pm 0.6	46 \pm 0.5	19.65 \pm 0.35
SA (10^{-5} mol/L)	4.2 \pm 0.06	68.0 \pm 2.1	62 \pm 1.0	60 \pm 1.1	22.73 \pm 0.75
SA (10^{-6} mol/L)	3.8 \pm 0.07	55.0 \pm 1.5	51 \pm 1.1	52 \pm 0.6	20.05 \pm 0.40
LSD _{0.05}	0.22	5.0	4.5	3.38	0.73

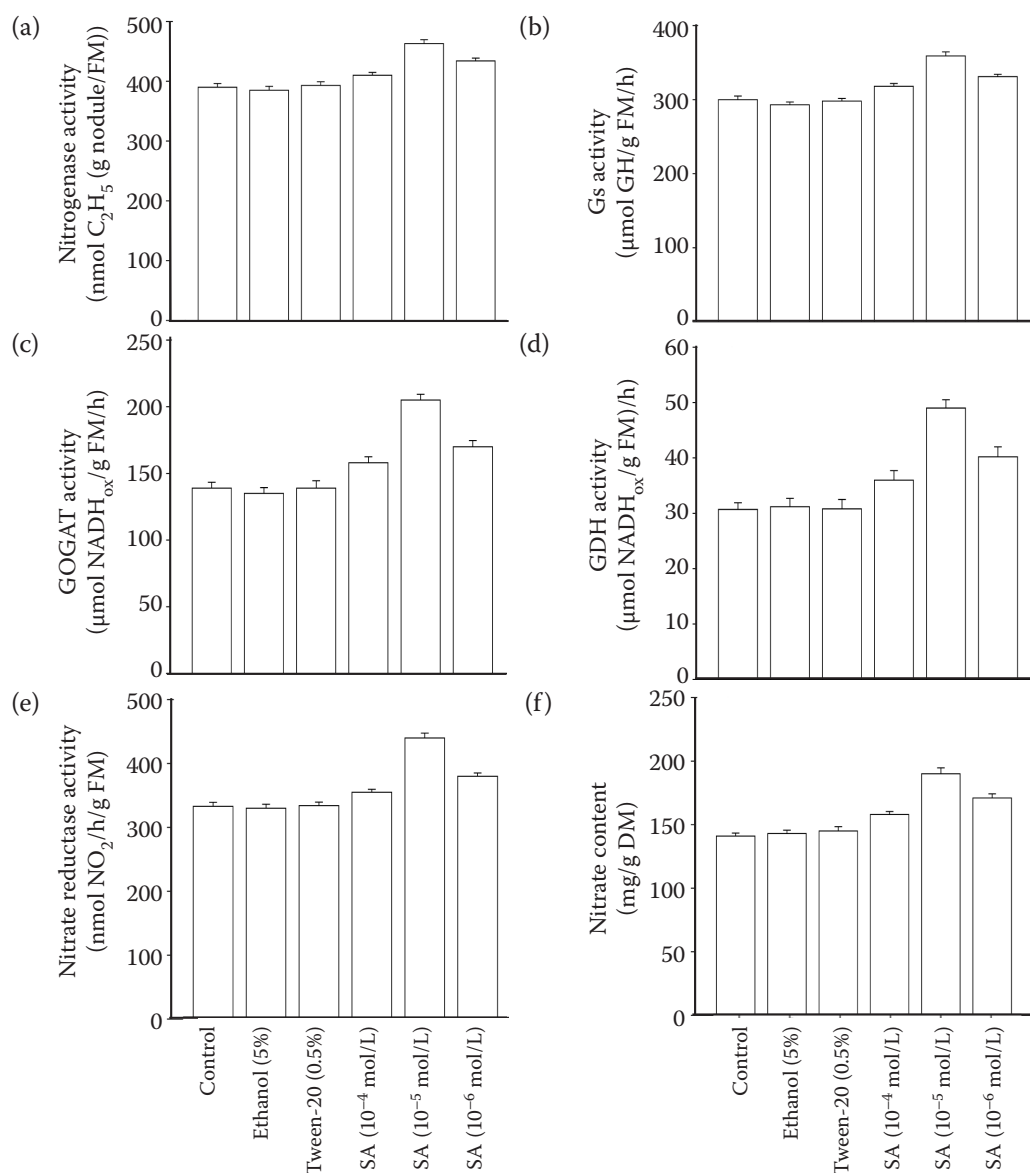


Figure 1. Effect of ethanol (5%), Tween-20 (0.5%) and different concentrations (10^{-4} , 10^{-5} or 10^{-6} mol/L) of salicylic acid on nitrogenase activity (a), glutamine synthetase (GS) activity in nodules (b), glutamate synthase (GOGAT) activity in nodules (c), glutamate dehydrogenase (GDH) activity in nodules (d), leaf nitrate reductase activity (e) and nitrate content in roots (f) of *Cicer arietinum* at 90 days after sowing. Data are the mean of three independent replicates. Vertical bars represent standard error (\pm)

Nitrate content in roots. Spraying of the plants with SA (irrespective of the concentration used) generated a significant increase of nitrate content in roots (Figure 1f). 10^{-5} mol/L proved to be statistically superior to other two concentrations as well as to that of control, where a significant increase of 34.7% and 40.6% was recorded for nitrate content in roots and nitrogen content in leaves over control. The response generated by the foliar application of ethanol or Tween-20 generated a response that was statistically equal to that of the control (Figures 1f and 2a).

CA activity and photosynthetic parameters. The activity of CA in leaves and photosynthetic

parameters (stomatal conductance and net photosynthetic rate) increased in response to the exogenous application of SA (Figures 2b–d). However, out of the three concentrations of SA, maximum response was generated in the plants sprayed with 10^{-5} mol/L of SA, showing a statistically significant increase of 60.0% (CA), 20.6% (g_s) and 46.92% (P_n) over that of the control. While comparing the effects of SA concentrations, the order of response was found to be 10^{-5} mol/L > 10^{-6} mol/L > 10^{-4} mol/L > control.

Antioxidant enzyme activities and proline content. Exogenous application of SA increased the activity of antioxidant enzymes and proline

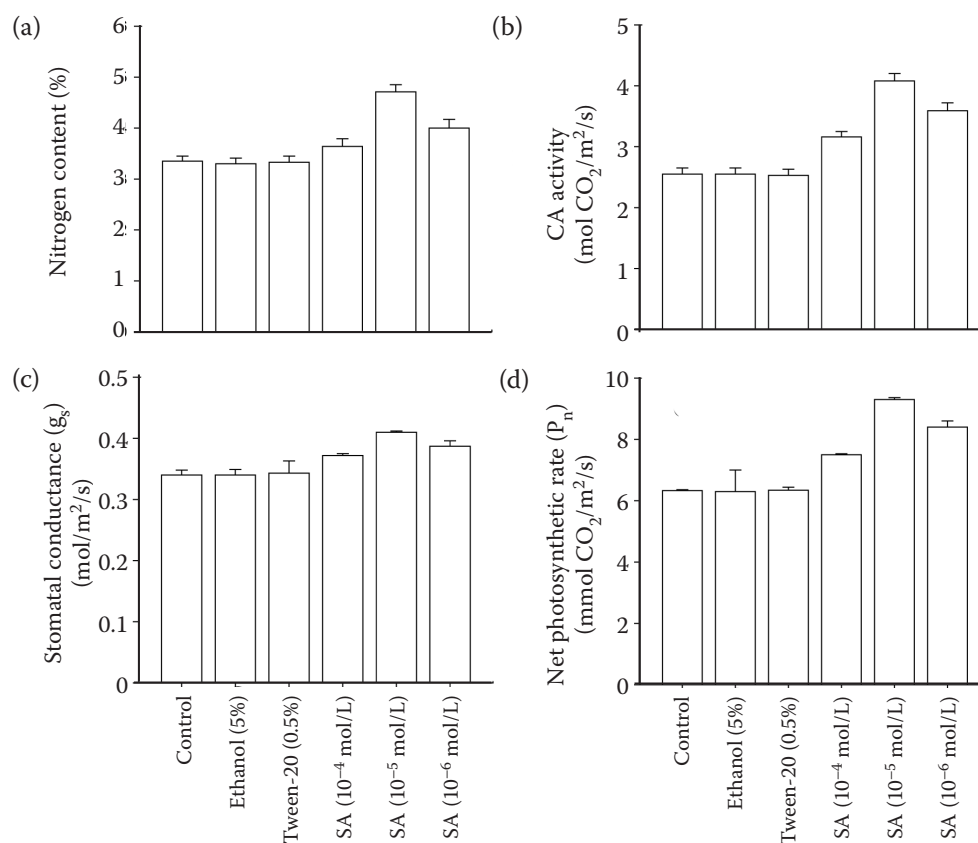


Figure 2. Effect of ethanol (5%), Tween-20 (0.5%) and different concentrations (10⁻⁴, 10⁻⁵ or 10⁻⁶ mol/L) of salicylic acid on nitrogen content (a), carbonic anhydrase (CA) activity (b), stomatal conductance (g_s) (c) and net photosynthetic rate (P_n) (d) in *Cicer arietinum* at 90 days after sowing. Data are the mean of three independent replicates. Vertical bars represent standard error (±)

content, where maximum response was generated in the plants sprayed with 10⁻⁵ mol/L of SA, showing a significant increase of 20.1% in the case of catalase (CAT); 45.2% in the case of guaiacol peroxidase (POX), 44.1% in case of the superoxide dismutase (SOD) and 43.1% (proline) over control. Ethanol or Tween-20 application did not generate any significant response (Figures 3a–d).

DISCUSSION

Several reports were published in the last decade demonstrating the role of SA applied as seed soaking treatment on various physiological processes. It was shown that the pre-soaking of pea seeds in SA had a beneficial effect on growth and photosynthesis, and led to a decrease in the oxidative injuries caused by heavy metal stress (Hayat et al. 2010). It was also noted that a dramatic inhibition of the germination process was reported above a concentration of 1 mmol in maize (Guan and Scandalios 1995) and *Arabidopsis* (Rajjou et al. 2006) plants. However, a complete understanding of the effect of exogenous SA applied as a foliar

spray is still lacking. It therefore becomes necessary to study the effect of varying concentrations of SA on plant growth and development. The effectiveness of SA applied as foliar spray depends on the type of species, time of application and the concentration used (Hayat et al. 2010). The reported research was undertaken to improve our understanding of the effect of the various concentrations of SA applied as foliar spray on the growth, photosynthesis, nitrogen fixation and its assimilation in chickpea and to find out the most effective concentration of SA.

Exogenous application of various concentrations (10⁻⁴, 10⁻⁵ or 10⁻⁶ mol/L) of SA resulted in an increased growth and physiological responses where the best response was generated by the 10⁻⁵ mol/L of SA. Similar results were obtained in *Brassica juncea* (Fariduddin et al. 2003) and in wheat (Hayat et al. 2005). The foliage of the plants sprayed with 10⁻⁵ mol/L of SA exhibited higher activity of NR (Figure 1e). However, a lower NR activity was observed at the SA concentration of SA of 10⁻⁴ mol/L. The possible reason for this concentration based effect of SA on NR activity is that NR activity was induced and/or degrada-

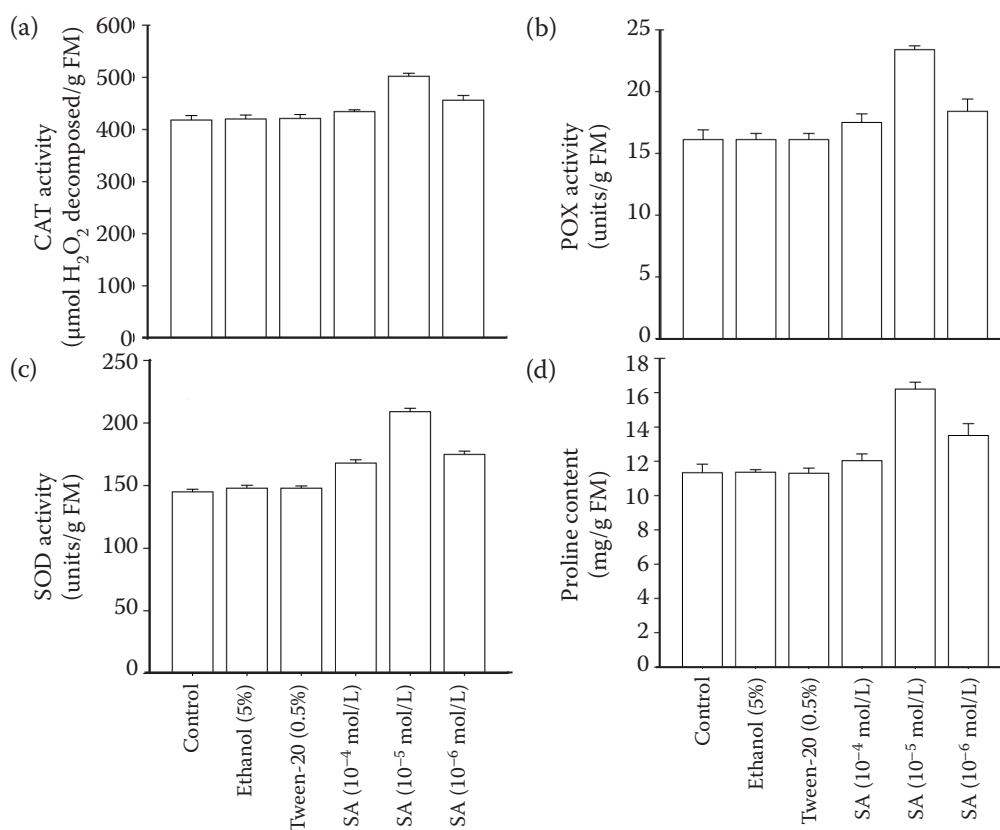


Figure 3. Effect of ethanol (5%), Tween-20 (0.5%) and different concentrations (10^{-4} , 10^{-5} or 10^{-6} mol/L) of salicylic acid on catalase (CAT) activity (a), peroxidase (POX) activity (b), superoxide dismutase (SOD) activity (c) and leaf proline content (d) in *Cicer arietinum* at 90 days after sowing. Data are the mean of three independent replicates. Vertical bars represent standard error (\pm)

tion of enzyme was prevented. Results indicated that concentration of SA at 10^{-5} mol/L might induce NR synthesis by mobilization of intracellular NO_3^- , and provide protection to in vivo NR degradation in absence of NO_3^- (Singh et al. 1997). It is also reported that low concentrations of SA increased NR activity while higher concentrations were found to be inhibitory in *Brassica juncea* (Fariduddin et al. 2003). Moreover, the content of any active protein (enzyme) represents a fine balance between its synthesis/activation and degradation/inactivation (Jain and Srivastava 1981). The concentration of SA might play an active role in such a regulation where the lower concentration favored an increase in the NR protein and higher quantity of SA decreased it (Figure 1e) by affecting the above processes. Moreover, the reason that seems to be most appropriate to explain the SA mediated elevation in the activity of NR is that it stabilizes the membrane structure and its fluidity which could have facilitated the increased uptake of nutrients including nitrate thereby increasing its content in the roots (Figure 1f) which also acts as an inducer of NR (Campbell 1999). The increase

in the content of nitrates and thereby activity of NR due to exogenous SA treatment under normal growth conditions was reported earlier (Hayat et al. 2005) which strongly supports the present results.

CA is the other most abundant soluble zinc containing enzyme in chloroplasts of C_3 plants, after Rubisco and facilitates the diffusion of CO_2 across the chloroplast membrane by catalyzing the hydration of dissolved CO_2 as it enters the most alkaline environment of stroma (Majeau and Coleman 1994) where CA catalyzes the reversible hydration of CO_2 and maintains a constant supply of Rubisco. The content of the enzyme and therefore, its activity is under the fine regulation at the level of transcription and/or translation (Okabe et al. 1980). However, either of these processes was favoured by the 10^{-5} mol/L of SA that increased the activity of CA (Figure 2b). The increased CA activity in the leaves is naturally expected to increase the photosynthetic efficiency and thereby the P_n (Figure 2d) by maintaining the constant supply of CO_2 for reduction by Rubisco (Okabe et al. 1980) as exogenous SA is known to increase stomatal conductance (Figure 2c) (Waseem et al.

2006). Further, exogenous SA is also known to increase pigment concentration, activate Rubisco and PEP carboxylase (Singh and Usha 2003). The improvement of all these characteristics ultimately increased P_n (Figure 2d). The increased photosynthesis is naturally expected to increase the growth of plants which was reflected in the form of increased dry mass per plant (Table 1). This increased activity of CA coupled with increased P_n will eventually result in increased production of photosynthates (carbohydrates). These carbohydrates are translocated in bulk to the nodules for the use of rapidly metabolizing bacteria which is consistent with the increased carbohydrate content in nodules (Table 1). However, the higher concentration of SA might have resulted in a permanent change at the level of membrane organization of the cells that proved to be injurious for plant's general metabolism and thereby reducing its overall growth and photosynthetic attributes (Table 1 and Figures 2c–d) which is further supported by the findings of Uzunova and Popova (2000).

The application of lower concentration of SA elevated the activity of antioxidant enzymes viz. CAT, POX and SOD (Figures 3a–c) which is in conformity with others (Hayat et al. 2008). This increase in the activity of antioxidant enzymes might be due to the regulatory role of SA at the level of transcription and/or translation. SA application is known to increase the endogenous proline content (Hayat et al. 2010) which is in conformity with the results of the present study where exogenous application of SA increased the *de novo* synthesis of proline thereby increasing its content in leaves (Figure 3d). Proline besides acting as an excellent osmolyte is also known for stabilizing the complex II electron transport (Hamilton and Heckathorn 2001), membranes and 3-D structure of proteins (Holmström et al. 2000) and enzymes such as Rubisco and CA (Mäkelä et al. 2000) thereby increasing photosynthetic attributes (Figures 2c–d). Thus the exogenous application of 10^{-5} mol/L of SA resulted in the increase in endogenous proline level (Figure 3d), whereas higher concentration might have reversed the phenomenon.

The sequence that leads to the establishment of nitrogen fixing mature nodules begins with the infection thread formed by the joint action of bacteria and host, which is activated by phytohormones (Hopkins 1995). Exogenous application of SA favours the legume-*Rhizobium* symbiosis (Hayat et al. 2010) thereby leading to the enhanced establishment and development of nodules which

is expressed in terms of increased number and dry mass of nodules (Table 1). The healthy nodule development and increased leghemoglobin content will obviously lead to increased activity of nitrogenase (Figure 1a), an oxygen labile enzyme which is protected by leghemoglobin. All these processes together bring about an efficient nitrogen fixation expressed in terms of increased nitrogen content (Figure 2a).

The nitrogen fixing potential of each nodule is determined by three main factors (Cooper and Scherer 2012): (a) photosynthates availability, which was maintained by enhanced photosynthesis (Figure 2), (b) low oxygen supply to the bacteroid, which at excessive level inhibits nitrogenase, was maintained by restricted O_2 supply by the mediation of increase in leghemoglobin (Table 1) and (c) export of fixed nitrogen in the form of ammonia. Nitrogen fixed in the form of ammonia diffuses across the peribacteroid membrane to the host cytosol by simple diffusion (Udvardi and Day 1990). Here two enzymatic systems, (a) GDH and (b) GS, GOGAT are operative to further metabolize it. GDH causes direct reductive amination of α -ketoglutarate, giving glutamate, whereas, GS catalyses the addition of NH_4^+ to glutamate forming corresponding amide, glutamine. This glutamine is converted back to glutamate by transfer of amide group to a molecule of α -ketoglutarate (Hopkins 1995). Since SA acts at transcriptional and/or translational level (Hayat et al. 2010), therefore might have accelerated the synthesis and thereby the activity of GDH, GS and GOGAT (Figures 1b–d). Further, the activity of enzymes GS, GOGAT and GDH might also be increased by lower concentration of SA through the involvement of auxins, as auxins are known to enhance the activity of GS, GOGAT and GDH (Hayat et al. 2009).

The results of the present study indicate that the application of 10^{-5} mol/L SA as a foliar spray increased the nitrogen fixation and assimilation of the chickpea plants. It also resulted in higher net photosynthetic rate which will probably increase the productivity of the crop.

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Corresponding author:

Associate Prof. Shamsul Hayat, Ph.D., Aligarh Muslim University, Department of Botany,
Plant Physiology Section, Aligarh, India
e-mail: hayat_68@yahoo.co.in
