Exogenous proline application enhances the efficiency of nitrogen fixation and assimilation in chickpea plants exposed to cadmium

Mohammed Nasser Alyemeni, Qaiser Hayat1, Shamsul Hayat*, Mohammad Faizan1 and Ahmad Faraz1

Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia.

Received: 05-06-2015 Accepted: 16-12-2015

ABSTRACT

Seeds of chickpea were sown in the pots supplemented with 0, 25, 50 or 100 mg of cadmium per kg of soil. At the stage of 30 days after sowing (DAS), the raised plants were sprayed with 20 mM proline except for the control plants which received double distilled water (DDW). The increasing degree of damage caused by the increasing concentration of Cd in soil was partially overcome by proline application. The treatment of 25 mg Cd fed plants with 20 mM proline increased significantly the nodulation parameters, leghemoglobin and carbohydrate content, leaf nitrogen and root nitrate content, activity of enzymes nitrogenase (E.C 1.18.6.1), nitrate reductase (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) over that of the control. The value of these parameters was found to be at par with that of the control in the plants exposed to 50 mg Cd per kg of soil and also treated with 20 mM proline. However, the treatment was not found to be effective in alleviating the adverse effects of 100 mg Cd per kg of soil.

Key words: Cadmium toxicity, Chickpea, Nitrogen assimilation, Nitrogen fixation, Nodulation.

INTRODUCTION

The problem of heavy metal pollution is increasing day by day which is posing a great menace to our agricultural crops thereby decreasing the crop productivity. “Heavy metals” are defined as the metals having density higher than 5 g cm⁻³. Out of the various heavy metals present in the contaminated crop environment, Cd was selected for the present study as it is easily taken up by plants and accumulated to high levels in the aerial organs particularly in chloroplasts thereby disrupting chloroplast function by damaging its membrane, inhibiting the activities of the biosynthesis of chlorophyll and CO₂ fixation (Siedlecka et al. 1997). Various physiological processes may be altered, including growth retardation and plant-water relations (Hasan et al. 2009). Cd-induced generation of superoxide (O₂⁻) anions, hydroxyl (OH) radicals and H₂O₂ cause considerable membrane damage. Cd is known to alter the activity of various antioxidative enzymes (Hasan et al. 2009). Cd toxicity is reported to decrease the nodulation and the activity of nitrogen metabolizing enzymes thereby decreasing the nitrogen fixing ability of the plants (Hasan et al. 2009). Based upon the toxicity generated by Cd, it has been ranked at seventh number among the top twenty toxins (Yang et al. 1998).

However, plants when exposed to abiotic stress respond by accumulating a wide array of metabolites particularly amino acids like proline. At the time of stress, proline acts as a protein-compatible hydrotropealleviate cytoplasmic acidosis by maintaining appropriate NADP⁺/NADPH ratios compatible with metabolism (Hare and Cress 1997). Proline, a multifunctional amino acid, besides acting as an excellent osmolyte is also known for stabilizing sub-cellular structures such as proteins and cell membranes, scavenging free radicals, balancing cellular homeostasis and signaling events and buffering redox potential under stress conditions (Hayat et al. 2012). Keeping in view the diverse physiological roles played by proline, the present study was aimed to elucidate whether exogenous application of proline could improve the nitrogen fixing and metabolizing efficiency of chickpea plants exposed to varying doses of Cd? The hypothesis tested is that exogenous application of the proline will alleviate the damaging effects of Cd in plants and thereby enhance the nitrogen fixation and assimilation in them.

MATERIALS AND METHODS

Growth conditions: The certified seeds of Cicerarietinum L. cv. Avarodhi were purchased from the Chola Beej Bhandara, Aligarh, India. The seeds were surface sterilized
with 0.01% mercuric chloride solution followed by inoculation with *Rhizobium* and were sown in five sets of earthen pots (10 inch diameter) filled with sandy loam soil and farmyard manure (6:1) arranged under a simple randomized block design. At the start of the experiment, Out of these five sets of prepared pots, four sets were supplemented with different doses (0, 25, 50 or 100 mg per kg of soil) of Cd, respectively and one set of pots was left untreated serving as control. At the stage of 30 DAS, the foliage of the resulting plants was sprayed with 20 mM proline, except control which received DDW instead of proline. The concentration of proline was selected on the basis of our earlier experiments (Hayat, 2010). The plant samples were collected at 60 and 90 DAS to assess various parameters. All the parameters studied followed a similar trend at both the sampling stages, however, the magnitude of the data for these parameters was higher at 90 DAS, and therefore, the data at this stage are shown in the present study. The methods adopted to assess each parameter are described below in the following pages.

**Nodule number and their fresh and dry mass:** The whole mass of soil was removed from the pot and placed in a bucket filled with tap water. The plants were moved gently to collect the intact root system with no damage to the nodules. The roots were then washed under running tap water and the number of nodules per plant was counted. The nodules were picked and weighed to record their fresh mass. Subsequently they were transferred to Petri-plates for overnight drying in an oven at 80°C. This dried material was weighed to obtain the dry mass of nodules per plant.

**Nodule Leghemoglobin content:** The leghemoglobin content in fresh nodules was estimated following the method described by Sadasivam and Mannickam (1992). Fresh nodules (200 mg) were mixed with 3 ml of 0.1M phosphate buffer and macerated in a mortar and pestle followed by filtration through two layers of cheese cloth. The nodule debris was discarded. The turbid reddish brown filtrate was centrifuged at 4,000 rpm for 10 minutes. Three ml of alkaline pyridine reagent was added to 3 ml of nodule extract and mixed properly. The solution becomes greenish yellow due to formation of hemochrome. The hemochrome was divided equally into two test tubes. To one test tube, a few crystals of potassium hexacyanoferrate were added to oxidize the hemochrome and read at 539 nm on spectrophotometer (spectronic 20D, Milton Roy, USA). To the other test tube a few crystals of sodium dithionite were added to reduce the hemochrome. This mixture was read at 556 nm after an interval of 2-5 minutes, against a reagent blank. The leghemoglobin content (mM) was calculated using the formula:

\[ \text{Lb concentration (mM)} = \frac{A_{556} - A_{539}}{23.4} \times 2D \]

Where: D is initial dilution

**Nitrate content in roots:** The nitrate content in roots was estimated following the method of Singh (1988). Root powder was macerated with acetic acid and was reacted with a powder mixture containing citric acid, manganese sulfate monohydrate, sulphanilamide, N-1-naphthyl-ethylenediamine hydrochloride and zinc. The absorbance of the reaction mixture was read at 540 nm. The absorbance of each sample was compared with that of the calibration curve plotted by using potassium nitrate. Nitrate content was computed on dry mass basis.

**Nitrogenase activity in nodules:** Nitrogenase activity was assayed adopting the procedure of Hardy *et al.* (1968). Assays were carried out immediately after harvesting the plants. Nodulated roots were cut from the base and were shaken slowly to remove attached soil particles. Samples were assayed in 30 ml glass tubes sealed with a subseal to allow it to be pierced by a hypodermic needle bearing a syringe. Five ml (v/v) of air was withdrawn from the sample container and replaced by an equal volume of acetylene gas. After an incubation of 1h at room temperature, 0.5 ml of gas was injected into a gas chromatograph (Nucon Series 5500) equipped with a flame ionization detector to detect the ethylene gas. The results were expressed in terms of nano moles of ethylene formed/g nodule fresh mass/hour.

**Nitrogen content:** The nodule nitrogen content was estimated by employing the method of Lindner (1944). The plant material was digested with concentrated H\(_2\)SO\(_4\) followed by neutralizing it with NaOH and sodium silicate solutions. Nessler’s reagent was added to this solution and samples were read on spectrophotometer.

**Nodule carbohydrate content:** Carbohydrates were estimated by adopting the procedure of Dubois *et al.* (1956). Dried nodule powder (50 mg) was transferred to a glass centrifuge tube containing 5 ml of 1.5 N H\(_2\)SO\(_4\). The sample was centrifuged at 4,000 rpm for 10 minutes. The supernatant was decanted into 25 ml volumetric flask with two washings of the residue with DDW. The volume was made up to the mark by using DDW. Out of this extract, 1 ml was placed in a test tube to which 1 ml of 5% distilled phenol was added. The test tube was placed in chilled water and 5 ml of H\(_2\)SO\(_4\) (AR grade) was added. The absorbance was read at 490 nm on a spectrophotometer. A blank was run simultaneously with each set of samples. Standard curve was plotted by using known graded dilutions of glucose solution. The absorbance of each sample was compared with the calibration curve and per cent carbohydrate content was calculated on a dry mass basis.

**Nitrate reductase activity:** The activity of NR was measured following the method laid down by Jaworski (1971). A mixture of fresh leaf samples, phosphate buffer (pH 7.5), KNO\(_3\) and isopropanol was incubated at 30°C
for two hours. Sulphanilamide and N-1-naphthyl ethylenediamine hydrochlorides solutions were added to the incubated mixture. The absorbance was read at 540 nm on a spectrophotometer.

**Assay of the activities of GS, GOGAT and GDH:** The extraction and assay of GS, GOGAT and GDH was done following the method described by Thimmaiah (1999). Extract for determination of GS activity was prepared from nodules homogenized in extraction buffer containing potassium phosphate buffer, sucrose, dithiothreitol, KCl, MgCl$_2$, and EDTA. Fifty ml of this enzyme extract was added to a reaction mixture containing Tris-maleate buffer, hydroxylamine, L-glutamine, ATP and EDTA. The absorbance was read at 540 nm on a spectrophotometer and was compared with a calibration curve plotted by using pure $\gamma$-glutamylhydroxamate. Extracts for determination of GOGAT and GDH activities were prepared from nodules homogenized in extraction buffer containing Tris HCl, sucrose and $\beta$-mercaptoethanol. One ml of this extract was added to the reaction mixtures consisting of Tris-HCl buffer, 2-oxoglutarate, NADH and NH$_4$Cl or L-glutamine for GOGAT and GDH, respectively. The absorbance was read at 340 nm on a UV spectrophotometer (Elico, India).

**Statistical analysis:** Each observation was replicated three times. The treatment means were compared by analysis of variance using SPSS software version 10 (SPSS, Chicago, IL, USA). Least significant difference (LSD) was calculated at 5% level of probability. Standard error (SE) due to replicates was also calculated.

**RESULTS AND DISCUSSION**

**Nodulation:** The spray of 20 mM proline to the foliage of unstressed plants resulted in a significant increase in nodule number (38.9%), nodule fresh mass (29.1%) and nodule dry mass (50.0%), over their controls (Figure 1a-c). A foliar spray of 20 mM proline to the plants fed with Cd (25 mg), increased the values of nodule number (14.8%), nodule fresh mass (9.8%) and nodule dry mass (20.0%) compared to the control. Further, spraying of plants with 20 mM proline also alleviated the ill effects generated by Cd (50 mg), bringing the values statistically equal to the control. However, the plants supplemented with Cd (100 mg) and also sprayed with 20 mM of proline possessed significantly lower values compared to the control (Figure 1a-c).

**Leghemoglobin content in nodules:** A significant increase of 32.0% over that of the control was observed in leghemoglobin content of unstressed plants, sprayed exogenously with proline, whereas the same spray to the plants fed with 50 mg Cd revealed the values for leghemoglobin content that were statistically equivalent to that of control. Spraying of proline to the foliage of the plants grown in soil supplemented with 25 mg Cd per kg of soil, resulted in a significant increase in the said parameter by 8.0% over control. However, on the other hand, the leghemoglobin content in the nodules of the plants fed with Cd (100 mg) and also sprayed with 20 mM of proline possessed significantly lower values compared to the control (Figure 1d).

**Nitrate content in roots:** The exogenous application of 20 mM proline significantly increased the nitrate content by 24.1% in unstressed plants and 7.6% in plants exposed to Cd (25 mg) over that of the control. Whereas, plants receiving Cd (50 mg) and also sprayed with 20 mM proline possessed significantly equal value for nitrate content with respect to control (Figure 1e). However, the nitrate content in the roots of the plants fed with Cd (100 mg) and also sprayed with 20 mM of proline possessed significantly lower compared to the control (Figure 1e).

**Nitrogenase activity in nodules:** The nitrogenase activity increased significantly by 22.9% over control, in the unstressed plants sprayed exogenously with 20 mM proline (Figure 1f). The application of proline to the foliage of plants...
that received Cd (25 mg) resulted in a significantly enhanced activity of nitrogenase over that of the control. Further, the proline treated plants, previously fed with Cd (50 mg), exhibited the nitrogenase activity comparable to that of the control plants at both the sampling stages. However, the proline spray proved to be inefficient in alleviating the negative effects generated by Cd (100 mg), where a significant drop of 7.1% in the nitrogenase activity was recorded when compared with the untreated control.

**Leaf nitrogen content:** The foliar application of 20 mM proline significantly increased the value of nitrogen content by 42.0% in unstressed plants and 8.6% in plants exposed to Cd (25 mg) over control (Figure 2a). Further, the plants which received Cd (50 mg) and were sprayed with 20 mM of proline also exhibited significantly higher value for leaf nitrogen content at both the sampling stages compared to the control (Figure 2a). However, the leaf nitrogen content was found to be 13.1% lower compared to the control.

**Carbohydrate content in nodules:** A significant increase of 17.9% in nodule carbohydrate content was observed in unstressed plants sprayed with 20 mM of proline compared to the plant grow under Cd stress condition. The stress generated by Cd (25 mg) was completely overcome and the values were increased by 8.6% over that of the control. Whereas, the plants fed with Cd (50 mg) and also sprayed with 20 mM of proline possessed statistically equivalent value of carbohydrate content to that of control at both the sampling stages. However, the carbohydrate content in the nodules of the plants fed with Cd (100 mg) and also sprayed with 20 mM of proline possessed significantly lower values (16.8%) compared to the control (Figure 2b).

**Nitrate reductase (NR) activity in leaves:** The exogenous application of 20 mM proline enhanced that NR activity by 29.3% (unstressed plants) and 14.8% (25 mg Cd fed plants) over their respective controls, whereas, the activity of this enzyme in plants exposed to 50 mg Cd and also sprayed with 20 mM of proline was found to be statistically at par with that of the control. However, the plants fed with Cd (100 mg) and also sprayed with proline showed a significant reduction of 8.2% in NR activity compared to control (Figure 2c).

**GS, GOGAT and GDH activities in nodules:** The activity of the enzymes GS, GOGAT and GDH followed the same pattern as was observed in case of nitrogenase activity. The unstressed plants sprayed with 20 mM proline, exhibited significantly higher activity of enzymes GS (20.6%), GOGAT (38.4%) and GDH (42.5%) in nodules, compared to the control, (Figure 2d-f). Further, foliar application of 20 mM proline to the plants grown in soil contaminated with 25 mg Cd, significantly increased the activity of enzymes by 8.0% (GS), 17.4% (GOGAT) and 19.0% (GDH) over that of control, whereas, the plants fed with Cd (50 mg) and also sprayed with 20 mM of proline, possess statistically equal value to that of control. However, the plants exposed to Cd (100 mg) and also received proline as foliar spray possess significantly lower value for GS (19.9%), GOGAT (7.2%) and GDH (17.5%) compared to the control (Figure 2d-f).

There is a homeostatic equilibrium between genetic makeup of a plant species and its habitat as dictated by the course of evolution. Any disturbance in the natural environment disturbs this equilibrium thereby causing serious physiological and metabolic perturbation in them which forces the plant to grow under stress. Among various stress factors, the problem of heavy metal accumulation in environment is on an increase which is posing a great menace to our agricultural crops thereby decreasing the crop productivity.

The foliage of the plants exposed to Cd stress exhibit a decline in the activity of NR which may be due to inhibition and/or metabolic dysfunction of the enzyme protein (Hopkins 1995). It is also known that the metal such
as Cd has a negative impact on the activity of plasma membrane bound ATP (Obata et al., 1996) and also the fluidity of membranes (Meharg, 1994), which may restrict the uptake of nitrate (Campbell, 1999) and thereby decreases the nitrate content in roots and consequently the activity of NR (Fig. 1 e and 2 c). Proline in combination with other analogues is known to increase the total phenolic content in plants (Kwok and Shetty, 1998) and phenolics are known to prevent auxindegradation (Schneider and Wightman, 1974). The increased level of auxins may increase the activity of NR, as exogenous supply of IAA increases NR activity (Hayat et al., 2009) which is evident from figure 2c.

Since proline acts as a membrane stabilizer (Hayat et al., 2012) it therefore, accounts for maintaining the nutrient status including nitrate content in roots. This report is in conformity with the increased nitrate content of roots following the exogenous application of proline (Figure 1e). Further, since nitrate is the inducer of NR (Campbell, 1999), therefore, the increased nitrate content might also be responsible for elevating the activity of NR (Figure 2c). This is further supported by the results obtained by Rajagopal (1981) in Vicia faba.

Cd brings about the closure of stomata by decreasing the partial pressure of CO$_2$ in the stroma (Hasan et al. 2009). However, the application of lower concentration of exogenous proline maintains a constant supply of CO$_2$ by increasing the stomatal conductance (Kamran et al., 2009), internal CO$_2$ and net photosynthesis (Ali et al., 2007). Proline is known to act as an enzyme protectant (Paleget et al., 1981). This is due the fact that 3-D structure of proteins (enzymes) is governed by the hydrophobic/hydrophilic interaction, ionic interaction and interaction between side chains of constituent amino acids. Proline could interfere with these side chains and thus play a protective role (Paleget et al., 1981) thereby increasing the activity of enzymes. A similar interaction might be operative between carbonic anhydrase (CA) and proline leading to the enhanced activity of CA. This increased activity of CA coupled with increased photosynthesis eventually results 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 (Figure 2b).

Cd is known to decrease the nodulation and the activity of nitrogen metabolizing enzymes thereby decreasing the nitrogen fixing ability of the plants (Hasan et al., 2008). Cd stress is also known to induce nodule senescence (Balestrasse et al., 2004) thereby decreasing the nodulation and leghemoglobin content which protects the O$_2$ labile enzyme nitrogenase, thereby decreasing its activity as well. The leghemoglobin breakdown might be a result of increased generation of ROS which is a characteristic feature of Cd toxicity (Balestrasse et al., 2004). However, the follow-up treatment with proline prevented the breakdown of leghemoglobin which may be due to the ROS scavenging potential of proline (Cuinad Shabala, 2007), thereby increasing the leghemoglobin content of nodules (Figure 1d) and thus increasing the activity of nitrogenase (Figure 1f). Another plausible reason for the increased nitrogenase activity might be that the exogenously applied proline is transported at a relatively faster rate across the symbiosome membrane, where it is metabolized by the bacteroids and used as an efficient source of energy for enhancing the nitrogenase activity in nodules (Pedersen et al., 1996). This is further confirmed by the increased pro-DH activity in nodules when proline was supplied exogenously (Kohl et al., 1991). This increased nitrogenase activity (Figure 1f) will obviously lead to the increased nitrogen fixation in the nodules which is exported to leaves leading to the observed increase in the leaf nitrogen content (Figure 2a). Further the nitrogenase activity might be regulated by proline at transcriptional and/or translational level. This notion is based upon the reports of King et al. (2000) who identified pro C gene which is essential for proline biosynthesis and concluded that this gene is essential for establishing symbiotic relationship between Bradyrhizobium japonicum and soybean. Cd stress retards the establishment of host-Rhizobium symbiosis thereby affecting nitrogen fixation (Rana and Ahmad, 2006). Since, exogenous proline favours the legume-Rhizobium symbiosis, it therefore favours nodulation which in present study is expressed in terms of increased number of nodules and consequently increases their fresh and dry mass (Figure 1a-c) by acting as an excellent source of energy. The nitrogen fixing potential of each nodule is determined by three main factors (Marschner, 2003); (a) photosynthates availability (b) low oxygen supply to the bacteroid, which at excessive level inhibits nitrogenase, which is maintained by restricted O$_2$ supply by the mediation of increase in leghemoglobin content 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 proline acts at transcriptional and/or translational level and also acts as an enzyme protectant (Paleg et al., 1981), therefore it might have accelerated the synthesis and thereby the activity of GS, GOGAT and GDH (Figures 2d-f).

Cd causes multiple direct and indirect effects on plant growth and metabolism (Hasan et al., 2009) by forming complexes with O, N and S ligands (Van Assche and Clijsters,
It interferes with mineral uptake (Yang et al., 1998) protein metabolism, membrane functioning, water relations and seed germination (Hasan et al., 2009). Moreover, they cause metabolic disturbance by altering essential biochemical reactions (Epstein and Bloom, 2005). However, at the same time, it is suggested that application of the lower concentration of proline increases the endogenous proline content under the heavy metal stress conditions which not only protects enzymes (Khedr et al., 2003), 3-D structure of proteins (Paleg et al., 1981), organelle and cell membranes by reducing the lipid peroxidation (Okuma et al., 2004) but also supplies energy for growth and survival, thereby helping the plant to tolerate stress (Hoque et al., 2007; Hayat et al., 2012).

**CONCLUSION**

It may be concluded from the present investigation that, the exogenous application of the 20 mM concentration of proline enhances the nodulation, nitrogen fixation and its assimilation in Cd stressed plants by neutralizing the ill effects generated by the presence of the metal in soil, thereby making proline an efficient tool to confer resistance against heavy metal pollution by protecting the plants nitrogen metabolizing machinery.

**ACKNOWLEDGEMENT**

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the research group project No. RGP-VPP-199.

**REFERENCES**


Hayat Q. (2010) Effect of proline and salicylic acid on the cadmium induced changes in chickpea (*Cicerarietinum L.*). Ph.D. thesis, Department of Botany, Aligarh Muslim University, Aligarh


