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Proline enhances antioxidative enzyme activity, photosynthesis and yield of *Cicer arietinum* L. exposed to cadmium stress

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Abstract – Seeds of chickpea inoculated with *Rhizobium* were sown in pots supplemented with different doses of cadmium (0, 25, 50 or 100 mg per kg of soil). At the stage of 30 days after sowing (DAS), the plants were sprayed with 20 mM solution of proline and were sampled at 90 DAS to assess the various parameters. The foliar treatment of proline resulted in the alleviation of the adverse effects generated by metal exposure, which was expressed in terms of the increase in plant growth. The activity of carbonic anhydrase in the cadmium-fed plants sprayed with proline was higher than that of control. The proline applied as foliar spray increased the photosynthetic attributes and yield characteristics in the cadmium-stressed plants. The activity of antioxidative enzymes increased with increasing concentration of cadmium. Maximum values were recorded in the plants exposed to 100 mg cadmium per kg of soil**.**

Key words: antioxidative enzymes, cadmium stress, *Cicer arietinum,* growth, photosynthesis, proline, yield

Introduction

Plant growth and development is under the fine control of the genetic information contained in a cell, where some genes are up-regulated and some genes are down-regulated. However, only a limited portion of this information is expressed at any given time of plant growth and development. There are certain factors that play a significant role in regulating the repression and de-repression of these genes. Out of these factors, light (THOMPSON and WHITE 1991), pollutants such as heavy metals (ROYALS et al. 1992), phytohormones (CLELAND 1999) and metabolites such as proline (ASHRAF and FOOLAD 2007) are noteworthy. Plants grow well in soil, not only because it provides them with anchorage but also

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with essential nutrients for their growth and development. Out of the various heavy metals, Cd is the most toxic. Although present in traces in soil, its level has enormously increased in recent times especially in areas of heavy automobile traffic, near smelters and by the use of sewage sludge, city refuse and Cd-containing phosphatic fertilizers. This increased concentration of Cd in soil causes growth inhibition associated with various metabolic dysfunctions and even plant death (HASAN et al. 2009). In order to cope with these conditions plants accumulate a wide array of metabolites, mainly proline (YANG et al. 2009). Proline, a multifunctional amino acid that 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 (SZABADOS and SAVOURE 2009). Proline may also function as a protein-compatible hydrotrope (SRINIVAS and BALASUBRAMANIAN 1995), alleviating cytoplasmic acidosis and maintaining appropriate $NADP^+$/NADPH ratios compatible with metabolism during stress (HARE and CRESS 1997).

Keeping in view the diverse physiological roles played by proline (HAYAT et al. 2012), the present study was aimed at elucidating whether exogenous proline could confer resistance against the damaging effects of Cd. The hypothesis tested is that exogenous application of proline will alleviate the damaging effects of Cd in plants and thereby enhance the growth, photosynthesis and yield attributes.

Materials and methods

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 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, and one set of pots was left untreated, serving as control. At 30 days after sowing (DAS), the foliage of the resulting plants was sprayed with 20 mM proline, except control which received double distilled water instead of proline. The concentration of proline was selected on the basis of our earlier experiments (data not shown). The plant samples were collected at 60 and 90 DAS to assess various growth and physiological 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 obtained at this stage of plant growth are presented in the present study.

Plant growth analysis and enzyme activities

The plants were uprooted and washed under running tap water. The root and shoot length of these plants was measured with the help of a graduated scale. These plants were blotted in blotting sheets to remove the adhering water and weighed on electronic balance to record their fresh weight. These plants were kept in an oven run at 80 °C for 72 hrs and then weighed to obtain their dry weight. The CA activity in the leaves was measured as described by DWIVEDI and RANDHAWA (1974). The leaf samples were cut in small pieces in cysteine

hydrochloride solution. These leaf samples were blotted and poured in a test tube, followed by addition of phosphate buffer (pH 6.8), 0.2 M $NaHCO_3$, bromothymol blue and methyl red indicator, at the last. This reaction was titrated against HCl. The activity of the enzyme was expressed on fresh mass basis.

For the assays of peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD) the leaf tissue was homogenized in 50 mM sodium phosphate buffer (pH 7.0) containing 1% soluble polyvinylpyrrolidone. The homogenate was centrifuged at 15.000 rpm for 10 min at 5 °C and the supernatant obtained was used as an extract for POX, CAT and SOD. The reaction mixture for CAT consisted of phosphate buffer (pH 6.8), 0.1 M H_2O_2 and 1.0 mL enzyme extract. H_2SO_4 was added to the reaction mixture, and after being incubated for 1 min at 25 °C, it was titrated against potassium permanganate solution (CHANCE and MAEHLY 1956). For the estimation of POX activity the enzyme extract (0.1 mL) was added to the reaction mixture consisting of pyrogallol phosphate buffer (pH 6.8) and 1% H_2O_2. The change in the absorbance was read for 2 min at the interval of 20 sec, at 420 nm on a spectrophotometer. The activity of SOD was measured according to BEAUCHAMP and FRIDOVICH (1971). A 3 mL of reaction mixture containing 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM nitroblue tetrazolium, 2 mM riboflavin, 0.1 mM EDTA and 0–50 mM of enzyme extract was prepared. Riboflavin was added last. This reaction mixture was exposed to low fluorescent light and the decrease in absorbance of the reaction mixture was read at 560 nm on a spectrophotometer. 50% inhibition was considered as one enzyme unit. The proline content in the fresh leaf sample was measured following the method described by BATES et al. (1973). Fresh leaf sample (0.5 g) was homogenized in a mortar with 5 mL of 3% sulphosalicylic acid. The homogenate was filtered through Whatman No. 2 filter paper and collected in a test tube with two washings each with 5 mL of sulphosalicylic acid. Two mL each of glacial acetic acid and acid ninhydrin were added to 2 mL of the above extract. This mixture was heated in a boiling water bath for 1 h. The reaction was terminated by transferring the test tube to an ice-bath. Four ml of toluene was added to the reaction mixture with vigorous shaking for 20–30 seconds. The chromophore (toluene) layer was aspirated and warmed to room temperature. The absorbance of red colour was read at 520 nm against a reagent blank.

Photosynthetic measurements, leaf water potential, yield characteristics and seed protein content

The stomatal conductance (g_s), intercellular CO_2 concentration (C_i), transpiration rate (E), water use efficiency (WUE) and net photosynthetic rate (P_N) in intact leaves were measured by LI-6400 portable photosynthesis system (LI-COR Lincoln, NE, USA), between 11:00 and 12:00 hrs under bright sunlight. The atmospheric conditions during measurement were photosynthetically active radiation (PAR), 1016±6 $\mu mol_{PHOTONS}\ m^{-2}\ s^{-1}$, relative humidity 60±3%, atmospheric temperature 22±1 °C and atmospheric CO_2, 360 mg L^{-1}. The ratio of atmospheric CO_2 to intercellular CO_2 concentration was constant. Leaf water potential, at each selected stage, was measured in fresh, detached leaves of the sample plants by using Psypro water potential system (Wescor Inc. USA). At harvest (160 DAS), 3 plants (replicates) from each treatment were randomly sampled and counted for the number of pods per plant. 25 pods from each treatment were randomly selected for computing the number of seeds per pod. The pods from three plants, representing each treatment, were

crushed, cleaned to assess the seed weight per plant. The total protein content in the dry seeds, at harvest, was estimated by the method of LOWRY et al. (1951).

Statistical analysis

The experiment was conducted according to randomized block design technique in the years 2009 and 2010. The data of both the experiments were pooled. Each treatment was represented by ten pots where each pot was considered as a replicate. Three observations (from three different pots) were recorded per treatment. 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.

Results

Growth characteristics

The spraying of plants with proline resulted in a significant increase in all the growth parameters, by 30.2% (root length); 29.8% (shoot length); 33.6% (plant fresh mass); 41.2% (plant dry mass), over that of the control (Fig. 1). The plants grown in 25, 50 or 100 mg of Cd per kg of soil possessed lower values for root length, shoot length, plant fresh and dry mass as compared to control and response of all the growth characteristics was dependent on the concentration of cadmium in the soil. All the growth characteristics (root length, shoot length, plant fresh and dry mass) decreased as the concentration of cadmium was increased from 25 to 100 mg of Cd per kg of soil. Further, the exogenous application of proline completely alleviated these ill effects generated by 25 mg of Cd per kg of soil on all the growth characteristics. However, the plants grown in the soil supplemented with 50 mg of Cd per kg soil and also sprayed with proline revealed values for all the growth characteristics that were not statistically different from those of control. However, the plants fed with Cd at the rate of 100 mg per kg of soil and also sprayed with proline, showed significantly lower values for these parameters than the control.

Carbonic anhydrase (CA) activity

The CA activity in unstressed plants was significantly increased (40.9%) by the foliar application of proline as compared to that of control (Fig. 2). The activity of CA in plants fed with Cd supplemented at the rate of 25 mg per kg of soil and also sprayed with proline was found to be 18.9% higher. However, the application of proline to the foliage of plants fed with Cd (50 mg per kg of soil) generated the values of CA that were statistically equal to that of the control. The activity of CA in plants exposed to Cd supplied at the rate of 100 mg per kg of soil and also sprayed exogenously with proline possessed significantly lower values (16.1%) than the control.

Photosynthetic parameters

Exogenous application of proline to the unstressed plants resulted in significant increases of 24.0% (g_s), 20.3% (C_i), 64.4% (WUE), 29.0% (E), 22.9% (P_N) as compared to the untreated control (Fig. 2). Further, the foliar application of proline alleviated the stress generated by Cd (25 mg per kg of soil) where a significant increase of 10.9% (g_s), 11.8% (C_i), 26.7% (WUE), 8.1% (E) and 11.2% (P_N) was recorded, compared to the control. The

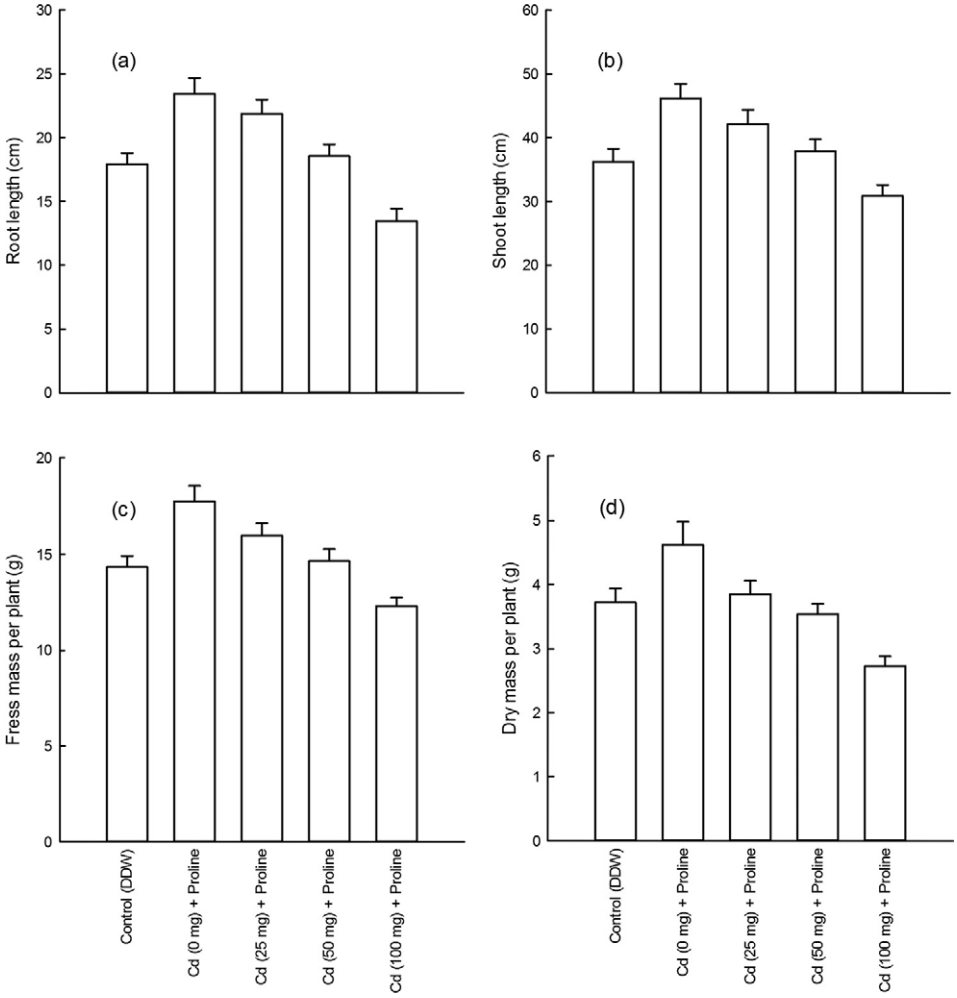

Fig. 1. Effect of proline on the cadmium-induced (0, 25, 50 or 100 mg Cd per kg of soil) changes in root length (a), shoot length (b), fresh mass (c), dry mass (d) in *Cicer arietinum* at 90 DAS. Data are the mean of three independent replicates. Vertical bars represent the standard error (±).

values of almost all these photosynthetic parameters, in the plants fed with Cd supplied at the rate of 50 mg per kg of soil, were found to be statistically equal to those of the control. However, like other parameters, the foliar application of proline to the plants receiving Cd (100 mg per kg of soil) showed significantly lower values for photosynthetic attributes, than the control (Fig. 2).

Antioxidative enzyme activities

The foliar application of proline to unstressed plants significantly enhanced the antioxidative enzyme activity by 11.9% (CAT), 25.0% (POX), and 26.4% (SOD), over that of the control (Fig. 3). The exogenous application of proline to the Cd fed plants further

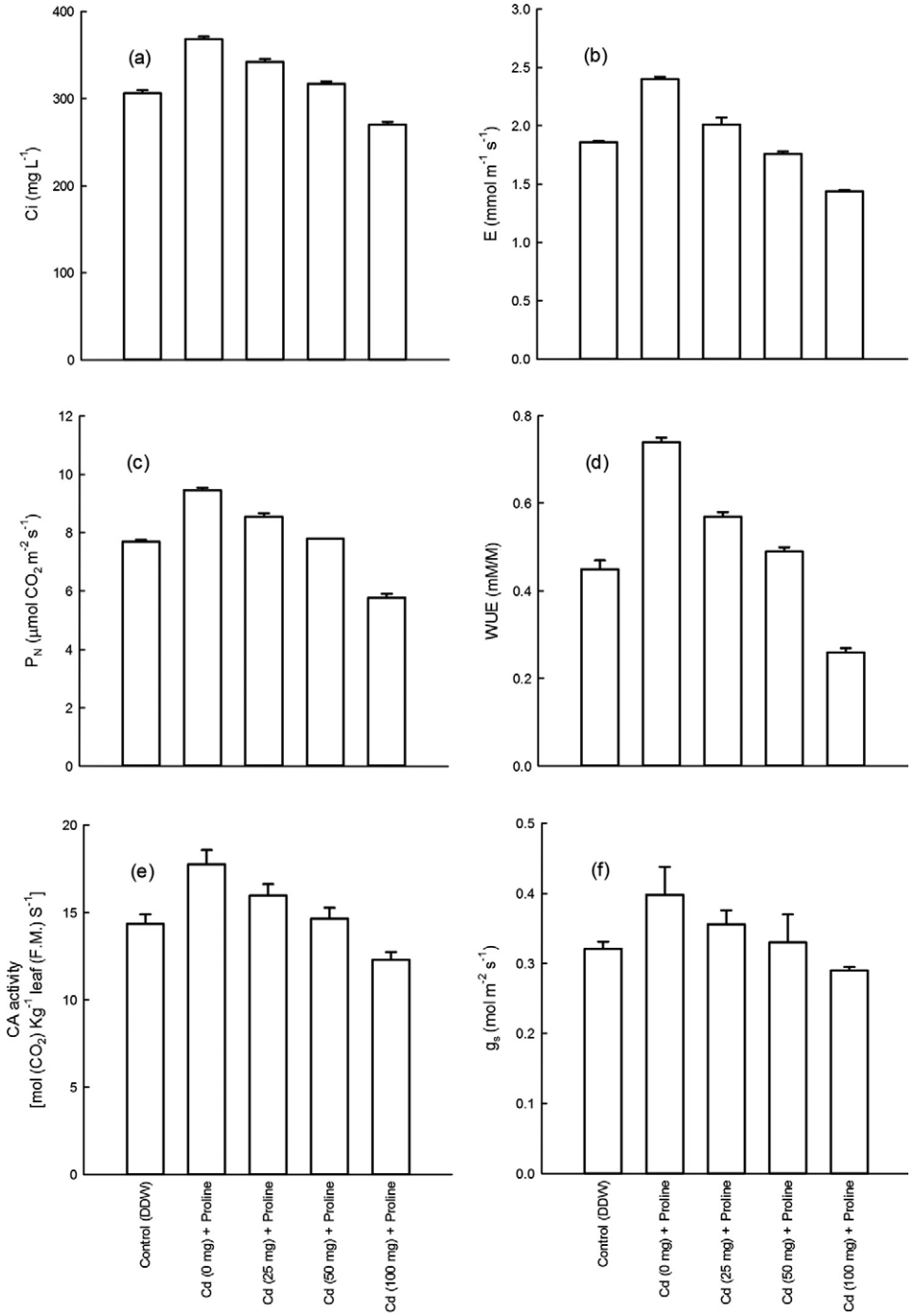

Fig. 2. Effect of proline on the cadmium-induced (0, 25, 50 or 100 mg Cd per kg of soil) changes in C_i (a), E (b), P_N (c), WUE (d), CA activity (e) and g_s (f) in *Cicer arietinum* at 90 DAS. Data are the mean of three independent replicates. Vertical bars represent the standard error (±).

elevated the activity of these enzymes over unstressed plants sprayed with proline as well as of the control plants. The treatment resulted in a significant increase of 17.1% (CAT), 42.8% (POX), 31.4% (SOD) in plants fed with Cd supplied at the rate of 25 mg per kg of soil; 24.1% (CAT), 57.9% (POX), 42.1% (SOD) in those fed with Cd (50 mg) and 31.5% (CAT), 82.9% (POX) and 61.4% (SOD) in the plants receiving 100 mg Cd per kg of soil, respectively, over that of the control (Fig. 3).

Proline content

The foliar application of proline significantly increased the endogenous proline content in the unstressed plants by 38.7%, compared to control (Fig. 4a). Further the endogenous

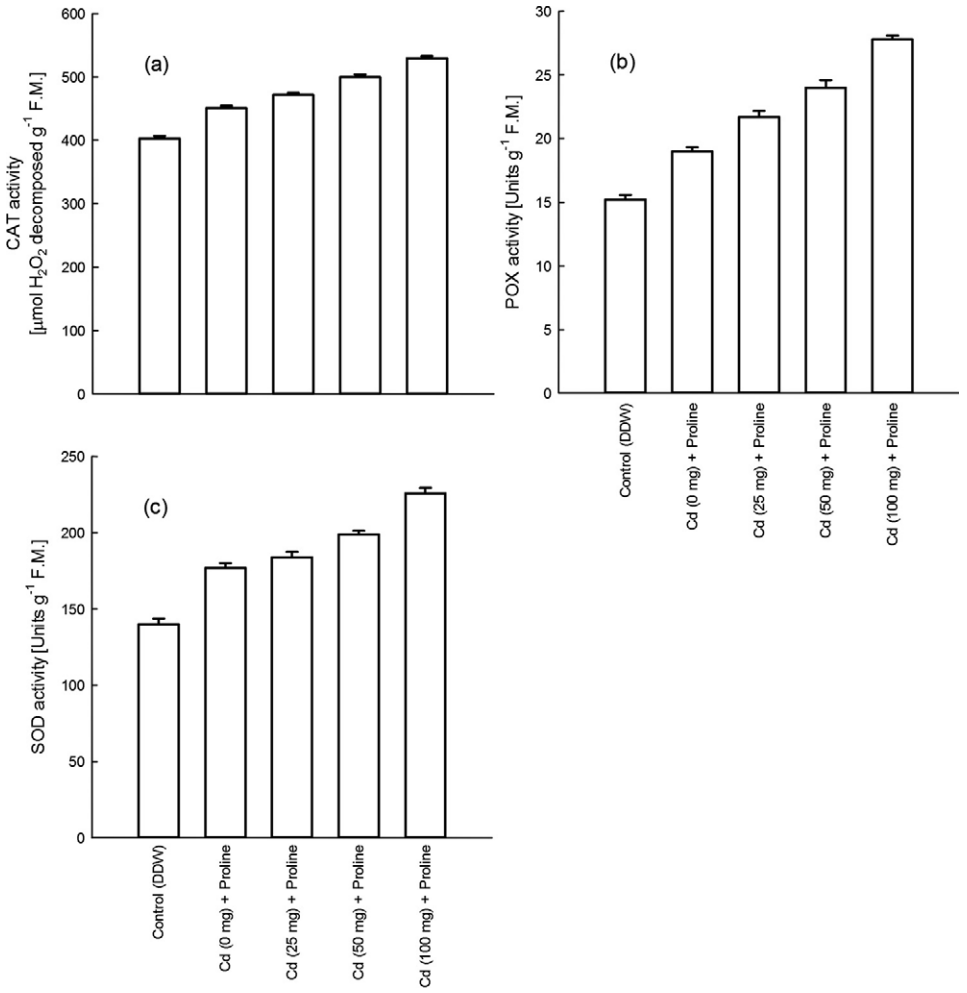

Fig. 3. Effect of proline on the cadmium-induced (0, 25, 50 or 100 mg Cd per kg of soil) changes in CAT (a), POX (b) and SOD activities (c) in *Cicer arietinum* at 90 DAS. Data are the mean of three independent replicates. Vertical bars represent the standard error (±).

proline level experienced a sharp increase in the Cd-fed plants when proline was sprayed on their foliage. The endogenous proline level in plants fed with 25, 50 or 100 mg Cd per kg of soil and sprayed with proline was increased significantly by 52.0%, 59.2% and 81.1% over their respective controls.

Leaf water potential

As is evident from figure 4(b), the leaf water potential increased significantly by 58.8%, compared to the control, in the unstressed plants sprayed with proline. The application of proline to the foliage of plants exposed to Cd (25, 50 or 100 mg per kg of soil) also resulted in a significant increase of leaf water potential over control. (Fig. 4b).

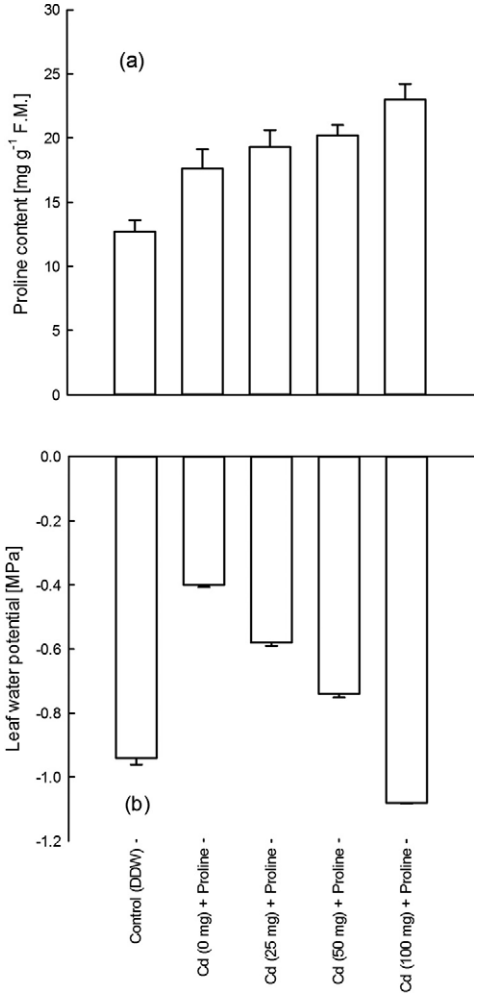

Fig. 4. Effect of proline on the cadmium induced (0, 25, 50 or 100 mg Cd per kg of soil) changes in proline content (a) and leaf water potential (b) in *Cicer arietinum* at 90 DAS. Data are the mean of three independent replicates. Vertical bars represent the standard error (±).

Number of pods per plant and number of seeds per pod

The exogenous application of proline to the unstressed plants and to those fed with 25 mg of Cd per kg of soil significantly enhanced the number of pods per plant, by 50.0% and 25.8% respectively, compared to the control (Tab. 1). Further the number of pods was found to be statistically equal to control in the plants exposed to Cd (50 mg per kg of soil) and also sprayed with proline, whereas it was significantly lower in the plants exposed to Cd (100 mg per kg of soil). On the other hand, the number of seeds per pod was found to be statistically non-significant, irrespective of the treatments.

Tab. 1. Effect of proline on the cadmium-induced (0, 25, 50 or 100 mg Cd per kg of soil) changes on yield characteristics and seed protein content in *Cicer arietinum*.

Treatments	Pod number	Number of seeds aper pod	Seed yield (g per plant)	mass of 100 seeds (g)	Seed protein content (%)
Control	19.40	2	5.80	15.55	18.55
Cd (0) + Proline	29.10	2	10.94	20.51	20.63
Cd (25) + Proline	24.40	1.33	7.81	16.63	19.81
Cd (50) + Proline	19.61	2	5.10	14.00	18.44
Cd (100) + Proline	16.49	2	3.51	10.00	16.75
LSD = 0.05	1.4	NS	0.5	1.0	0.7

N.S. – Non significant

Seed yield and mass of 100 seeds

The seed yield and mass of 100 seeds also followed the same pattern observed in number of pods per plant. A significant increase of 88.6% (seed yield) and 31.9% (mass of 100 seeds) was recorded in unstressed plants sprayed with proline (Tab. 1). Foliar spray of proline on the plants exposed to Cd (25 mg per kg of soil) also resulted in a significant increase of 34.7% (seed yield) and 7.0% (mass of 100 seeds) over control.

Seed protein content

The application of proline to the foliage of unstressed plants and to those exposed to 25 mg Cd per kg of soil, increased the seed protein content significantly, by 11.2% and 6.8%, respectively, over that of control (Tab. 1). Further, the proline spray onto the plants exposed to 50 mg Cd per kg of soil revealed values for seed protein content that were statistically equal to that of the control. However, the value of seed protein content in the 100 mg Cd-fed plants and also treated with proline was found to be statistically lower than that of the control.

Discussion

Cadmium causes multiple direct and indirect effects on plant growth and metabolism by forming complexes with O, N and S ligands and also inhibits net photosynthesis in various crops (Hasan et al. 2009). Cadmium becomes associated with the cell wall and middle

lamellae, which increases the cross linking of pectins and results in the inhibition of cell growth (BARCELO and POSCHENREIDER 1990) and therefore, the plant as a whole. However, the follow-up treatment with proline improved the growth of plants expressed in terms of increased length of root and shoot and fresh and dry mass of the plant (Fig. 1). The concentration of proline was based on our preliminary experiment in which 10, 20, 30, 40, 50 mM of proline was tested (HAYAT and HAYAT 2011). In view of some earlier reports (HAYAT et al. 2012), it is suggested that the application of proline increases endogenous proline content (Fig. 4a) under heavy metal stress conditions. This will help to protect enzymes (ISLAM et al. 2009), 3-D structure of proteins (PALEG et al. 1981) and organelle and cell membranes by reducing the lipid peroxidation (OKUMA et al. 2004). Besides this it also supplies energy for growth and survival, thereby helping the plant to tolerate stress (HOQUE et al. 2007) thereby enhancing growth characteristics (Fig. 1).

Cd brings about the closure of stomata by decreasing the partial pressure of CO_2 in the stroma (BARCELO and POSCHENREIDER 1990), which becomes a direct cause of the reduction of the g_s, C_i, WUE and E. Further, Cd is known to enhance the activity of the enzyme chlorophyllase, which brings about the degradation of chlorophyll (REDDY and VORA 1986) and also reduces the synthesis of δ-amino-levulinic acid and protochlorophyllide reductase complex (GADALLAH 1995), leading to decreased pigment concentration. The reduced photosynthesis in Cd-treated plants might also be due to the reduced activity of CA (HASAN et al. 2008). The activity of this enzyme is largely determined by photon flux density, concentration of CO_2, the availability of Zn (TIWARI et al. 2005) and gene expression (KIM et al. 1994). The stress generated by Cd decreases the partial pressure of CO_2 in the stroma by inducing the stomatal closure (BARCELO and POSCHENREIDER 1990) resulting in the loss of CA activity. However the follow-up treatment of Cd-stressed plants with proline resulted in enhanced CA activity and thereby increased P_N (Fig. 2). It is well documented that the exogenous application of proline protects plants against stress by stabilizing the complex II of electron transport chain in mitochondria (HAMILTON and HECKANTHORN 2001), membranes and 3-D structure of proteins (HOLMSTROM et al. 2000) and enzymes such as Rubisco and CA (MAKELA et al. 2000) thereby increasing photosynthetic attributes (Fig. 2). Therefore, the follow-up treatment with proline resulted in enhanced activity of these enzymes, which may be due to the ROS scavenging potential of proline (ZHOU et al. 2010) through enhancement of the activity of antioxidative enzymes (ISLAM et al. 2009) i.e. CAT, POX and SOD (Fig. 3) and the endogenous level of proline (Fig. 4a). These results are supported by previous findings that exogenous proline elevated the free proline content eliciting its biosynthetic pathways (HAYAT et al. 2012) and the activity of the antioxidative enzymes (CAT, POX and SOD) by acting at the level of transcription and/or translation. Since Cd causes membrane degradation by increasing the lipid peroxidation, thereby leading to significant electrolyte leakage (HASAN et al. 2009), it will obviously result in a lowering of leaf water potential (Fig. 4b). However, the follow-up treatment with proline enhanced the leaf water potential by protecting the membranes from metal induced oxidative damage (OKUMA et al. 2004) thereby decreasing the electrolyte leakage and increasing the leaf water potential (Fig. 4b). The increased growth, photosynthetic efficiency and elevated activity of antioxidative enzymes under the influence of exogenous proline are likely to increase the yield (Tab. 1). A plausible reason for the increase in yield is the delay of senescence of plant organs (particularly leaves and flowers) in response to exogenous proline (BALESTRASSE et al. 2004), which will help the plant to improve the efficiency of photosynthetically active

sites and also prevent the premature loss of flowers and fruits. This consequently resulted in the observed increase in the number of pods per plant (Tab. 1). Proline is known to increase the total phenolic content in plants (KWOK and SHETTY 1998) and phenolics are known to prevent auxin degradation (SCHNEIDER and WHITMAN 1974), which might be responsible for the increase in the seed yield. This gets additional support from the observations of HAYAT et al (2001) that phytohormones increase the degree of sink at the level of seeds, directing the flow of metabolites to the developing seeds thereby improving the mass of 100 seeds and consequently the seed yield per plant at harvest (Tab. 1).

Conclusion

It can be concluded from the present investigation that the foliar application of proline to cadmium-treated plants decreased the negative effects generated by the cadmium. All the growth and photosynthetic parameters increased significantly when proline was applied as foliar spray. The activity of antioxidative enzymes and the proline content in the Cd fed plants increased further with foliar spray of proline.

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