

ORIGINAL ARTICLE

**Studies on the effect of Cu (II) ions on the antioxidant enzymes
in chickpea (*Cicer arietinum* L) cultivars**

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Seed is a developmental stage that is highly protective against external stresses in the plant life cycle. Present study was undertaken with the aim to elucidate the toxic effect of Cu (II) ions on the antioxidant enzymes in chickpea cultivars during seed development and growth. Seven-day-old seedlings were subjected to different concentrations (0-100 ppm) of CuSO₄ · 7H₂O for 2 days followed by analysis of effect on different antioxidant enzymes. Our results indicated that with the increase in the Cu ion concentration, the antioxidant activities of catalase, ascorbate peroxidase and superoxide dismutase showed a differential behavioral pattern. Besides the antioxidant activity, the toxic effect was also observed in other physiological parameters *viz.* root/shoot length, RWC and lipid peroxidation. This study indicates that the toxicity of Cu stress is dependent on the physiological state of tissue surrounding the embryo is in part responsible for determining the toxicity.

Key words: Antioxidant enzymes, Chickpea, Copper sulphate stress, lipid peroxidation

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Key words: Antioxidant enzymes, Chickpea, Copper sulphate stress, lipid peroxidation

Rapid industrialization, urbanization and intensive agriculture increasing contamination of heavy metals in soil have become a major concern. Contamination of agricultural soil by heavy metals has become a critical environmental concern due to their potential adverse ecological effects. The primary response in response to heavy metals in plants is the generation of reactive oxygen species (ROS). Various metals viz. Cd, Cu, Zn, Ni, Co, Cr, Pb, As etc either generate ROS directly through Haber-

Weiss reactions or overproduction of ROS and occurrence of oxidative stress in plants could be the indirect consequence of heavy metal toxicity (Mithofer *et al.*, 2004; Yadav, 2010).

Among heavy metals, copper (Cu) has been classified as a non-biodegradable metal pollutant which enters the environment through various anthropogenic activities such as from pesticide, fungicides and municipal sewage (Ross, 1994). Copper toxicity has been shown to induce toxicity

which alters the photosynthetic and respiratory processes, enzyme activity, DNA, and membrane integrity by inducing lipid peroxidation, generates oxidative stress and ROS thereby causing disturbance of metabolic pathways and leading to growth inhibition (Alaoui-Sosse' *et al.*, 2004, Hegedus *et al.*, 2001). Accumulation of ROS activates antioxidative defense mechanisms in plants. The protection against oxidative stress is achieved by the production of enzymatic antioxidants which comprised of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) while glutathione, carotenoids and ascorbate represent non enzymatic components (Hall, 2002; Shao *et al.*, 2008). These biochemical attributes served as an index of metal sensitivity or tolerance in different groups of plants (Li *et al.*, 2006; Srivastava *et al.*, 2004).

Chickpea is the second most produced legume crop in the world after dry bean with 9, 7 million tons produced in 2007 (FAO 2007) and is indispensable to Indian agriculture as a predominant source of protein for vegetarian population. This study was aimed to investigate the role of high copper concentration on the growth parameters, biochemical parameters antioxidant enzymes in different cultivars of chickpea.

MATERIALS AND METHODS

Seed of two cultivars of chickpea variety (PBG-5 and PDG-4) were germinated in Petri dishes in a growth chamber at 25°C, 12 h light /12 h dark period, (illumination of 2500 Lux, Philips T2 40W/33 lamp). Seven-day old seedlings were subjected to viz. 20, 40, 60, 80 and 100 ppm concentrations of CuSO₄.5H₂O for 24 hours. The samples were harvested and stored at -20°C till further analysis

Relative water content (RWC) was estimated

according to the method of Castillo (1996) and calculated in the leaves for each drought period. Samples (0.5 g of the seedling tissue) were saturated in 100 ml distilled water for 24 h at 4°C in the dark and their turgid weights were recorded. Then they were oven-dried at 65°C for 48 h and their dry weights were recorded. RWC was calculated as follows: $RWC (\%) = [(FW - DW) / (TW - DW)] \times 100$, where FW, DW, and TW are fresh weight, dry weight and turgid weight, respectively.

Lipid peroxidation was measured in terms of content of malondialdehyde (MDA, $\epsilon = 155 \text{ mmol}^{-1} \text{ cm}^{-1}$), a product of lipid peroxidation, following the method of Heath and Packer (1968). 0.5 g seedling were homogenized in 10 ml 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000 g for 5 min. To 1 ml aliquot of supernatant, 4 ml 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) TCA was added. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000 g for 10 min, the absorbance of the supernatant was recorded at 532 nm. The value for nonspecific absorption at 600 nm was subtracted. MDA content was expressed as nmol MDA per g fresh weight.

The activity of catalase were analysed using the method described by Abej, 1984. The assay mixture consist of 50 μL of the enzyme extract, 100 mM phosphate buffer (pH 7.0), 0.1 μM EDTA, and 20 mM H₂O₂ in a total volume of 1.5ml. The decrease of H₂O₂ was monitored by reading the absorbance at 240 nm at the moment of H₂O₂ addition and 1 min later. The difference in absorbance (DA_{240}) was divided by the H₂O₂ molar extinction coefficient (36 $\text{M}^{-1} \cdot \text{cm}^{-1}$) and the enzyme activity expressed as $\text{mmol of H}_2\text{O}_2 \text{ min}^{-1} \cdot \text{mg}^{-1} \text{ protein}$.

Specific APX activity was assayed using the assay mixture consisted of 50 μL of the enzyme extract,

50 mM phosphate buffer (pH 6.0), 0.1 μ M EDTA, 0.5 mM ascorbate, and 1.0 mM H_2O_2 in a total volume of 1.5 ml as per the method described by Nakano and Asada (1981). Ascorbate oxidation was monitored by reading the absorbance at 290 nm at the moment of H_2O_2 addition and 1 min later. The difference in absorbance (DA_{290}) was divided by the ascorbate molar extinction coefficient ($2.8 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) and the enzyme activity expressed as $\text{mmol of } H_2O_2 \text{ min}^{-1} \cdot \text{mg}^{-1} \text{ protein}$, taking into consideration that 1.0 mol of ascorbate is required for the reduction of 1.0 mol of H_2O_2 .

SOD activity was determined according to the method described by Roth and Gilbert, 1984. One millilitre of reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 100 mM EDTA, 20 ml enzyme extract and 10 mM pyrogallol. The enzyme activity [U (mg protein)⁻¹ 21] was calculated by monitoring the reaction mixture for 120 s (at 60 s intervals) at 420 nm on a Nanovue[®] device.

RESULTS

We investigated the role of Cu(II) ions on the growth of seedlings in different cultivars of chickpea. It was observed that with the increasing concentration of $CuSO_4 \cdot 7H_2O$ from 20 ppm to 100 ppm, a gradual decrease from 8-40% in shoot length was obtained when compared in cultivar PDG-4 as compared to 20-50% decrease in shoot length in cultivar PBG-5 under similar set of $CuSO_4$ concentrations. Similarly the growth of roots in these cultivars also get affected by the Cu stress where it was observed that a decrease of 10-55% in the root length was observed in both the cultivars (Table 1)

MDA formation is considered as the general indicator of lipid peroxidation (Wang and Zhou, 2006). Exposure to Cu resulted in an accumulation

of lipid peroxidation products in leaves of both the cultivars of chickpea (Figure 1). It was found that in cv. PDG-4 the MDA activity increased from 20-60 ppm from 89-105% followed by a slight decrease at 100 ppm conc. however a significant increase from 108-116% was observed at 20-60 ppm Cu(II) ion conc. followed by a decrease at higher conc. of Cu stress ($P < 0.05$).

As observed in Figure 2 in cultivar PDG-4 maximum catalytic activity of catalase was observed at 80 ppm concentration and min. at 40 ppm concentration compared to control. CAT activity decreased by 70%, 31% as compared to control at 20 ppm, 40 ppm and 100 ppm concentration whereas increased to 62%, 108% at 60 ppm and 80 ppm concentration as compared to cultivar PBG-5, in which the activity of catalase increased significantly with the increase in stress concentration as compared to control. The maximum catalytic activity was observed at 60 ppm concentration. Increase in catalytic activity varied from 20% to 100%.

The effect of Cu stress was also observed for the Ascorbate peroxidase enzymes. Results showed that activity of ascorbate peroxidase decreased in both the PDG-4 and PBG-5 as compared to control plants. The activity of APX increased from 115-230% in PBG-5 as compared to 100-145% increase in cv. PDG-4) as compared to control under 20-100 ppm conc. of Copper stress (Figure 3). But still APX showed higher activity in PBG-5 as compared to PDG-4. The superoxide dismutase activity in PDG-4 first increased with the mild stress and showed highest activity at 60 ppm (136%) and then gradually decreased at higher concentrations where as in cv. PBG-5 a constant increase in the SOD activity of 149-170% was observed of the stress as compared to control plants (Figure 4).

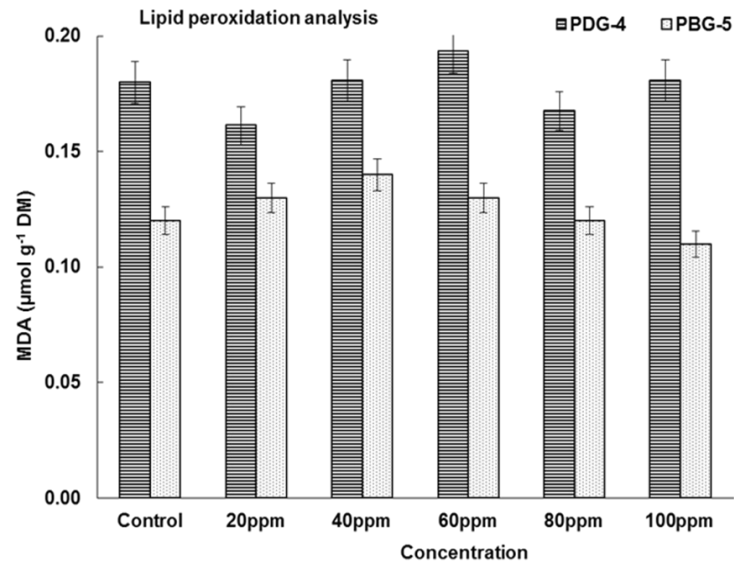


Figure 1. Effect of CuSO_4 stress on lipid peroxidation activity on two cultivars of chickpea. Vertical bars represent Standard deviation

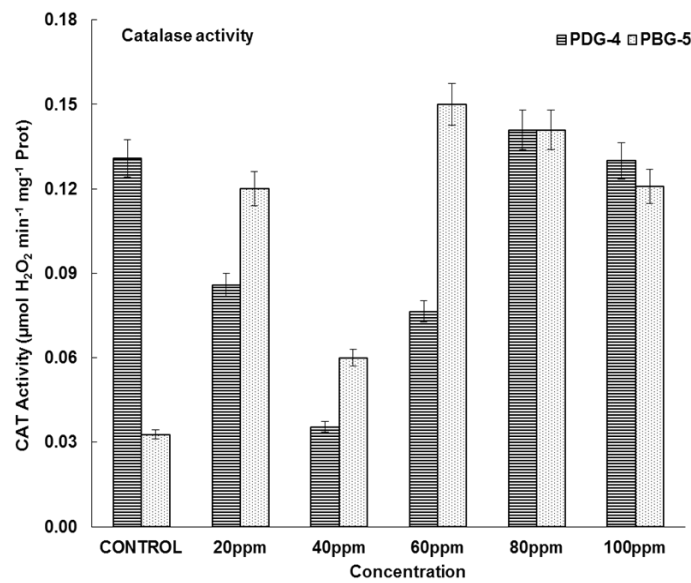


Figure 2. Comparative analysis of catalase activity on two cultivars of chickpea. Vertical bars represent Standard deviation

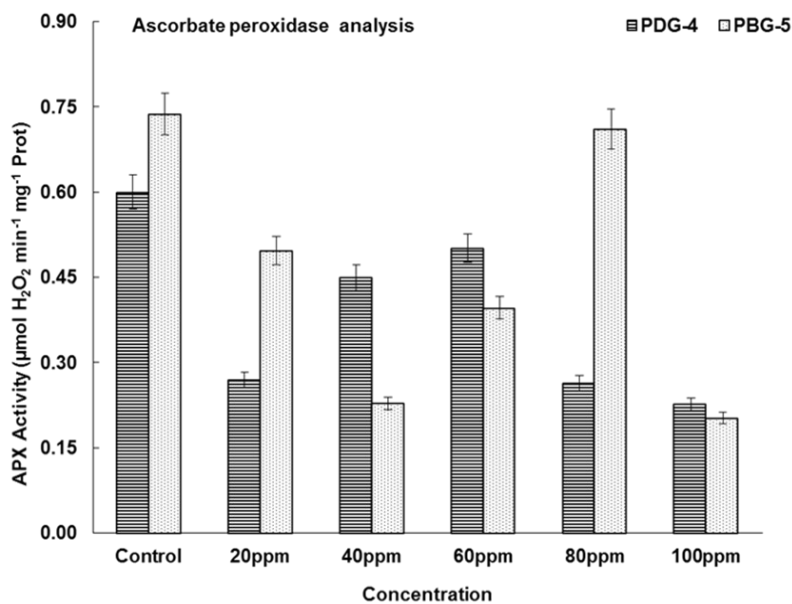


Figure 3. Effect of CuSO₄ stress on ascorbate peroxidase enzyme activity on chickpea cultivars. Vertical bars represent Standard deviation

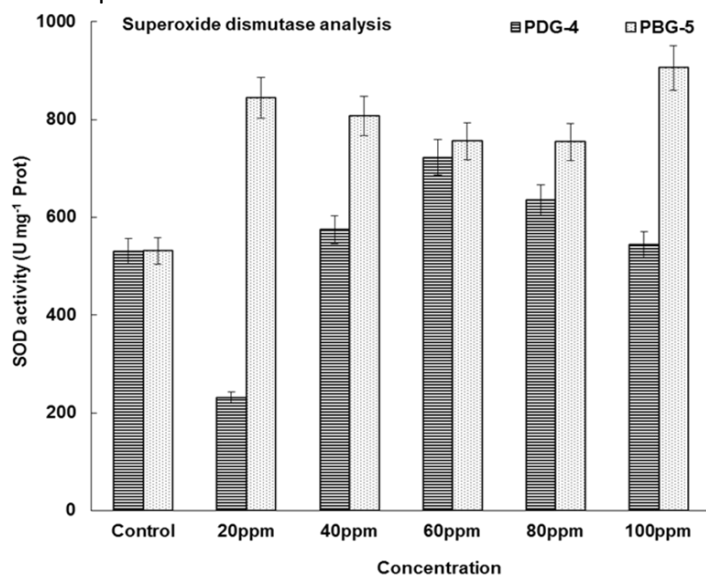


Figure 4. Comparative analysis of SOD activity on two cultivars of chickpea. Vertical bars represent Standard deviation

Table 1. Effect of Cu (II) ions on the root and shoot lengths in two cultivars of chickpea

Parameters	Shoot length (cm)		Root length (cm)	
	PBG-5	PDG-4	PBG-5	PDG-4
Control	16.3 ± 0.5	13.7±1.6	14.5±1.5	16.5 ±1.5
20 ppm	13.6 ±1.4 (20%)	12.2±0.8 (8%)	13.9±0.1 (13%)	13.8 ±1.2 (30%)
40 ppm	10.5±1.5 (30%)	11.5±1.5 (14%)	11.5±0.5 (25%)	11 ±1.0 (40%)
60 ppm	10.2±0.8 (36%)	10.1±0.9 (21%)	9.6±0.4 (30%)	9.2 ±0.5 (45%)
80 ppm	8.9±1.2 (45%)	9.3±1.7 (28%)	8.5±0.5 (45%)	7.5 ±1.5 (50%)
100 ppm	8.5±0.5 (50%)	8.5±1.5 (40%)	7.8±0.2 (50%)	6.8 ±0.2 (60%)

DISCUSSION

When a plant is subjected to any biotic or abiotic stress factor, the first observed response is a decrease in its normal metabolic activities, with a consequent reduction of growth. A gradual decrease in root (13% to 60%) and shoot elongation (8% to 50%) rate was observed with the increase in CuSO_4 concentrations in both the cultivars. It has been recorded that elevated copper concentration inhibit the normal growth and development of chickpea plants and this study is in agreement with the similar studies as in *Jatropha curcas* seedling (Gao *et al.*, 2008) and *Prunus cerasifera* plantlets (Lombardi *et al.*, 2005).

Lipid peroxidation is an important symptom of heavy metal toxicity, and has been reported in several species (Demiral and Türkan, 2005). We observed that lipid peroxidation rate in Cu exposed plants increased in both the cultivars as compared to control plants in a dose dependent manner with a maximum damage to the plants treated with 100ppm of Cu concentration. (Figure 2) A noticeable increase in MDA signifies oxidative stress since excessive copper has been reported to enhance the activity of lipoxygenase in various plants viz. soybean, *Artemisia annua* L. and in safflower (Ahmed *et al.*, 2010; Fernandez and Henriquez, 1991) which catalyzes lipid peroxidation, indicated by an increase in MDA level.

The ability of plants to overcome heavy metal stress relies on the induction of antioxidant enzymes like catalase, ascorbate peroxidase and superoxide dismutase (Alscher *et al.* 2002). The level of H_2O_2 in plant cells is under the control of CAT and POX, which can lower concentration if it is produced in excess. CAT eliminates H_2O_2 by breaking it down directly to form water and oxygen.

It is less efficient than POD in H_2O_2 scavenging because of its low substrate affinity. In flowering plants, there are three genes that encode CAT isoforms. External factors stimulate the transcription of these genes in a different way (Słomka *et al.*, 2008). APX, CAT activity showed the largest increase at the copper concentration of 60ppm but with increasing copper concentration it declined in cultivar PDG-4 but in cultivar PBG-5, it increased with the increase in stress concentration. These results are in agreement with the results of some other studies with several plant species like in *Vigna mungo* (Solanki *et al.*, 2011), *Brassica juncea* (Mobin and Khan, 2007) that suggest increased peroxidase activity in response to elevated copper concentrations.

SOD is an essential component of the antioxidative stress defence system in plants, catalyzing the dismutation of superoxide into oxygen and hydrogen peroxide (Mittler 2002). The results of this study regarding an increased SOD activity in both the cultivars in response to excess of copper are in agreement with those obtained in oat, wheat and *Arabidopsis thaliana* (Alscher *et al.* 2002, Draobzkiewicz *et al.* 2004) and *Prunus cerasifera* plantlets (Lombardi *et al.*, 2005). These findings suggested that SOD is involved in the oxidative stress defense in chickpea (Bowler *et al.*, 1992). We conclude that copper toxicity has a drastic role in the growth and development in plants and has resulted in poor growth and yield of chickpea seedlings. The data confirm the inhibitory effect of heavy metal stress on relative water content and shoot and root lengths which are subsequently slowed down by Cu treatments. The increase in lipid peroxidation activity was proportional to the increased doses of Cu stress. The most phytotoxic influence was observed at the

highest applied concentration (100ppm). However, to cope with heavy-metal toxicity, chickpea plants were able to promote the activities of antioxidant enzymes such as Catalase, Peroxidase, Superoxide dismutase and Ascorbate peroxidase.

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