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Antioxidant defence mechanisms in chickpea (*Cicer arietinum* L.) under copper and arsenic toxicity

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Chickpea (*Cicer arietinum* L.) is one of the oldest pulse crops cultivated from ancient time both in Asia and Europe. India is the largest producer of chickpea in the world, sharing 65 and 70% of production and total global area, respectively. The inhibition of plant growth and crop productivity by excess heavy metals is a global agricultural problem. Therefore, a pot experiment was conducted at wire house of the Department of Plant Physiology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi during *Rabi* season of 2009-10, using a complete randomized design with four replications and five treatments. Our experimental findings reveal that excessive amount of copper (Cu) (200 mg/kg) and arsenic (As) (200 mg/kg) in soil caused a mark reduction in nitrate reductase enzyme activity at both growth stages. However, a little increment was found in proline content, peroxidase and superoxide dismutase activity under higher doses of Cu and As at both growth stages. The study aimed to get more information on physiological changes in activity of antioxidant enzymes under heavy metal toxicity.

Key words: Antioxidant enzymes, chickpea, heavy metals, nitrate reductase, proline content.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the oldest pulse crops cultivated from ancient time both in Asia and Europe. India is the largest producer of chickpea in the world, sharing 65 and 70% of production and total global area, respectively. It is grown in tropical, sub-tropical and temperate regions as winter season crop. Chickpea contains 19-21% protein and 60% carbohydrate, so it provides an excellent quality of dietary protein for overcoming future food shortages. Heavy metals are metals with density higher than 5 g cm^{-3}. Fifty three out of the ninety naturally occurring elements have density higher than 5 g cm^{-3} but not all of them are of biological importance. Based on their solubility under physiological conditions, seventeen heavy metals may be available for living cells and are of importance to organism and ecosystem (Weast, 1984). Among these metals, Fe, Mo, Zn, and Mn are important as micro nutrient: Ni, Co, Cu, V, W and Cr are toxic elements with high or low importance as trace elements. However, As, Hg, Ag, Cd and Pb have no known function as nutrients and seem to

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be more or less toxic to plants and microbial population (Godbold and Hatlerman, 1985).Heavy metals enter the food chain from industrial sources, natural resources and geochemical cycle. Heavy metals are extremely phytotoxic in nature and the danger is aggravated by their almost indefinite persistence in the environment.

The inhibition of plant growth and crop productivity by excessive heavy metals is a global agricultural problem. The most widespread visual evidence of metal phyto-toxicity is reduction in plant growth as metal toxicity increases. However, different metals have different sites of action within the plant. Copper as heavy metal plays a vital role in deteriorating soil biotic and abiotic factor and disrupting plants metabolism. There are several factors that increase copper concentration up to toxic level in the soils such as soil chemical characteristics, agricultural manufacturing, metalliferous mining, smelting sites, waste disposal practices, use of metal containing pesticides, sewage sludge, etc.

Copper as a micronutrient plays a role in normal metabolism in higher plants; it participates in the reduction of electron transfer in photosynthesis in the form of plastocyanin. However, copper at high level becomes strongly phototoxic to cell and causes inhibition of plant or even death (Mocquot et al., 1996). Studies from plant species demonstrate that excess copper in plant growth medium induces formation of ROS in treated tissues; copper induced generation of hydrogen peroxide is directly correlated with damage to protein and lipids (Murphy and Taiz, 2000). Photosynthesis is also sensitive to excess copper; the pigment and protein components of photosynthesis membranes are the targets. Excess copper causes an elevated susceptibility to photo inhibition of Ps-II *in vitro* and *in vivo*. It has long been known that high concentration of copper when added to the incubation medium of isolated thylakoids inhibits PS-II electron transfer activity on the acceptor sites. In addition, copper toxicity is related to the disturbances in the uptake of essential elements (Assche and Clijsters, 1990). Arsenic can be potential activator of the biosynthesis of phytochelatins (pcs) in several plants (Maitani et al., 1996; Schmoger et al., 2000). So far, little has been known about its effect on the synthesis of homophy-tochelatins (hpcs) in plants. Work done by previous workers suggests that there is increase in oxidative stress under heavy metal toxicity. Therefore, there is need to study the effect of heavy metals such as copper and arsenic on important metabolic enzymes and antioxidant enzymes activity. Hence, an investigation was undertaken to study the antioxidant defence mechanisms in chickpea (*C. arietinum* L.) under copper and arsenic toxicity.

MATERIALS AND METHODS

The present investigation was carried out with a variety of chickpea (Radhey) obtained from the Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University Varanasi during *Rabi* season of 2009-10, using a complete randomized design with 4 replications and 5 treatments. The details of the treatment are: control, copper sulphate (100 and 200 mg/kg), sodium arsenate (100 and 200 mg/kg) soil. The seeds were sown in plastic pots containing 3 kg pulverized farm soil mixture with farm yard manure in the ratio of 3:1. The sowing was done on 26th November, 2009. Fertilizer was applied at the ratio of 20, 35 and 25 ppm of N, P and K, respectively. There were four pots for each replication and four healthy plants were kept in each pot. Antioxidant enzyme activities were done on two separate plants from each pot at two different growth stages through destructive techniques.

For estimation of nitrate reductase activity, leaves from uniformly grown seedlings in a homogeneous population were selected for enzyme induction. The enzyme activity was assayed *in vitro* by the method of Srivastava (1975) in the first fully expanded leaf. Peroxidase activity was assayed in leaf tissue at early stage after imposing stress in control and treated plants. The enzyme assay was performed according to the protocol of Kar and Mishra (1976). Superoxide dismutase activity was assayed in leaf tissue at early stage after imposing stress in control and treated plants using the method of Dhindsa et al. (1981). Proline content was determined in leaf sample after imposing stress in control and treated plants using the method of Bates et al. (1973).

Statistical analysis

Simple CRD design was followed in all observations. Analysis of variance was performed on the data at each stage as described by Panse and Sukhatme (1967). Least significant difference (LSD) was calculated at 5% probability level.

RESULTS AND DISCUSSION

Nitrate reductase activity

Significant and greater reduction in nitrate reductase (NR) activity was observed at higher doses of arsenic and copper (Table 1). Arsenic showed greater depression in NR activity as compared to copper but there was non-significant difference at the same doses of arsenic and copper. Sodium arsenate of 200 mg/kg recorded the least NR activity and significantly differed from control and lower doses of copper and arsenic. Non-significant differences were observed between copper sulphate of 100 mg/kg and sodium arsenate of 100 mg/kg at 30 and 45 days after sowing (DAS). There was 65.0% reduction in NR activity as observed in sodium arsenate (200 mg/kg) as compared to the control.

Proline content

Proline content increased from 30 DAS to 45 DAS under stressful environment (Table 1). Highest significant increase in proline content was observed in sodium arsenate of 200 mg/kg (65.8%) as compared to copper sulphate of 100 and 200 mg/kg. But copper sulphate of 100 and 200 mg/kg was at par at 30 DAS. Greater increase in proline content was observed at 30 DAS as

Table 1. Effect of copper and arsenic on nitrate reductase, peroxidise, proline and superoxide dismutase activity in chickpea at 30 and 45 days after sowing.

Treatment	Dose (ppm)	Nitrate reductase (µmoles NO_2^- g^{-1} fresh weight $hour^{-1}$)			Peroxidase (U g^{-1} fresh weight min^{-1})			Proline (µg g^{-1} fresh weight)			Superoxide dismutase (U g^{-1} fresh weight min^{-1})		
		I	II	Mean	I	II	Mean	I	II	Mean	I	II	Mean
Control	-	0.34	0.58	0.46	14.23	14.45	14.34	23.48	25.38	24.43	153.65	168.50	161.0
Copper Sulphate	100	0.21 (-38.2)	0.51 (-12.0)	0.36	15.38 (+8.0)	15.92 (+10.1)	15.65	32.36 (+37.8)	34.34 (+35.0)	33.35	186.80 (+21.7)	198.36 (+17.7)	192.5
Copper Sulphate	200	0.13 (-61.7)	0.15 (-74.0)	0.14	16.43 (+15.4)	17.13 (+18.5)	16.78	34.25 (+45.9)	36.28 (+42.9)	35.27	260.05 (+69.2)	291.96 (+73.2)	276.0
Sodium Arsenate	100	0.18 (-47.0)	0.38 (-34.0)	0.28	15.13 (+6.3)	15.56 (+7.6)	15.30	36.83 (+56.9)	38.94 (+53.4)	37.89	352.82 (+129.6)	380.89 (+126.0)	366.8
Sodium Arsenate	200	0.08 (-65.0)	0.09 (-84.0)	0.08	17.33 (+21.7)	17.87 (+23.6)	17.60	38.92 (+65.8)	41.29 (+62.6)	40.11	391.61 (+154.8)	410.05 (+143.3)	400.8
Mean		1.12	1.30	-	4.70	5.18	-	33.17	35.25	-	287.06	288.62	-
SEM±		0.03	0.05	-	0.33	027	-	1.52	1.04	-	8.32	7.3	-
C.D. at 5%		0.19	0.14	-	0.98	0.81	-	4.47	3.12	-	24.94	21.7	-

Figure in parenthesis represents percentage increase (+) and decrease (-) over control, I and II means data recorded at 30 and 45 days after sowing.

compared to 45 DAS except for sodium arsenate of 200 mg/kg. Arsenic and copper at higher doses resulted in higher production of proline in plants. 100 and 200 mg/kg copper sulphate and 100 mg/kg sodium arsenate did not show significant differences between them at both growth stages, but there was significant differences observed in the control.

Peroxidase activity

There was significant increase in peroxidase activity observed with increasing doses of arsenic and copper (Table 1). Mean peroxidase activity recorded at both growing stages was least in control (14.23 U g^{-1} fresh weight min^{-1}) and highest in sodium arsenate of 200 mg/kg (17.87 U g^{-1} fresh weight min^{-1}) in all the treatments. Higher doses of arsenate significantly differed from all other treatments with respect to peroxidase activity. Similarly, all the treatment varied significantly for peroxidase activity except control and copper sulphate of 100 mg/kg at 30 DAS. The highest increase in peroxidase activity was recorded in sodium arsenate of 200 mg/kg (23.6%) at 45 DAS as compared to the control.

Superoxide dismutase activity

Changes in superoxide dismutase (SOD) activity are presented in Table 1. SOD activity increased significantly with increasing doses of arsenic and copper at both growth stages. Arsenic had greater increase in SOD activity as compared to copper. There was significant difference for SOD activity in all the treatments at both growth stages. Highest increase in SOD activity was recorded in sodium arsenate of 200 mg/kg (54.8%) as compared to control. Mean SOD activity was recorded as least in control (153.65 U g^{-1} fresh weight min^{-1}) and highest in sodium arsenate (200 mg/kg) (410.05 U g^{-1} fresh weight min^{-1}).

DISCUSSION

The inhibition of plant growth and crop production by excess heavy metals such as copper, cadmium, arsenic, or lead in contaminated-soil is a global agricultural problem. In this study, an effort was made to study the effect of two highly toxic metal pollutants of soil (arsenic and copper) on different antioxidant enzymes activities in chickpea at 30 and 45 days after imposing the heavy metal stress treatment. It was observed that arsenic had more pronounced toxic effects

in terms of reduction in NR activity and increased proline content, peroxidase activity and SOD activity in comparison with copper at the same concentrations. Gupta et al. (2004) also reported more inhibitory effect of arsenic and cadmium on plants as compared to Cu, Zn and Ni. They have concluded that growth inhibition is specific for metal species rather than their total uptake by the tissues.

The presence of arsenic and copper significantly inhibited the activity of the enzyme nitrate reductase (NR) and the degree was dependent on the concentration of the metal used. This may be due to inhibition or metabolic dysfunction of the enzyme protein. Moreover, metals also have an impact on the activity of plasma membrane bound proton pump and the fluidity of the membrane restricting the uptake of nitrate, the inducer and the substrate of NR.

Arsenic and copper stress also resulted in an increase in the level of proline in the leaves of chickpea plant. There was more increase in proline content at higher doses of the heavy metal. The accumulation of free proline in response to heavy metal is widespread among plant. The accumulation of proline is a gene regulated process which is the consequence of the over expression of the gene involved in its biosynthesis and depression of those involved in its degradation, in the plants under stress. The functional significance of proline accumulation under heavy metal stress might include water balance maintenance, scavenging of hydroxyl radical or metal chelation.

The activity of two antioxidant enzymes peroxidase and superoxide dismutase (SOD) was found to increase on imposition of arsenic and copper stress. There was increased activity of these enzymes at higher doses of heavy metal stress. Heavy metals are known to cause the production of ROS, that is, the formation of superoxide (O_2^-), hydroxyl (OH^-) radicals, hydrogen peroxide (H_2O_2) and singlet oxygen (O_2) in plants (Foyer et al., 1997). Mohan et al. (1997) suggested that the increase of peroxidase activity may be an effect of accelerated senescence, connected with enhanced formation of hydrogen peroxide (H_2O_2) or secondary metabolites such as phenolic compound. It has been demonstrated in *Zea mays* that catalase and SOD were stimulated after exposure to arsenic (Mylona et al., 1998). Arsenic accumulated in the plant tissue stimulates proxidase synthesis during the early phases of plant development, long before the appearance of visible changes (Miteva and Peycheva, 1999; Stoena et al., 2003). There are also reports from several plant species establishing that Cu causes oxidative stress and increases the activities of anti-oxidative enzymes (Luna et al., 1994; Gupta, 1999).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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