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Studies on lipid peroxidation and antioxidant enzymes in the germinating seeds of *Cicer arietinum* exposed to cobalt

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Abstract

The effect of varying levels of cobalt on per cent germination, proline and protien content, lipid peroxidation, antioxidant enzymes was studied in the germinating seeds of chickpea (*Cicer arietinum* L.). The surface sterilized seeds, after soaking in 0, 50, 100, 150 or 200 µM of cobalt for 12 hours, were allowed to germinate in sterilized petri plate at 25 ± 2 ^{0}C, in an incubator. The germinating seeds, at 36 and 72 hrs after germination exhibited a significant decrease in relative water content, mean daily germination, germination percentage, protein content, nitrate reductase activity and antioxidant enzymes (catalase, peroxidase and superoxide dismutase). However, lipid peroxidation and proline level exhibited a linear increase in response to the treatments with passage of time. The seeds showed maximum inhibitory effect at 200 µM Co concentration.

Key words

Antioxidant enzymes, *C. arietinum*, Cobalt, Lipid peroxidation

Introduction

Seed germination in *sensu stricto* is a cumulative consequence of many physical, physiological, biochemical, cellular and molecular events rendering the radicle to emerge from the seed. They are well versed with intracellular bodies of lipids, proteins, carbohydrates, organic phosphate and various other inorganic compounds which facilitate the process of germination and growth of the resulting seedlings. These compounds are consumed during the course of germination by involving various enzymes such as hydrolases, lipases, proteinases and phosphatases are released and/or synthesized *de novo* to facilitate the availability of simpler substances to the embryo, for its growth (Bewley and Black, 1994). However, the industrialization and anthropogenic activities have resulted in the accumulation of different pollutants such as heavy metals, pesticides, and other potential toxicants in the environment. Besides various metabolic processes and growth, seed germination is also affected by stress, although the magnitude of effect may vary. Among heavy metals, cobalt (Co) enters the soil through application of sludge/effluents used by local farmers for irrigating their crops, thus introducing these pollutants into the food chain through these crops (Warning *et al.,* 1996). Apart from this, contamination by Co in soil has been reported due to discharge from several industries, metal refineries, and vehicular and aircraft exhausts (Barceloux, 1999) in addition to natural parent-rock sources. At a very low level, Co has been found to be beneficial for plants as it an integral component of the coenzyme cobalamine (vitamin B_{12} and its derivatives) (Marschner, 2003). Its deficiency has been found to affect nitrogen metabolism in legumes (Dilworth *et al.,* 1984). However, elevated concentration has been found to cause toxicity in plants. It causes a marked inhibition of growth together with chlorosis and necrosis, declines the Hill and catalase activity and deteriorates the quality of produce (Chatterjee *et al.,* 2006). Excess cobalt also causes inhibition of enzymes related to chlorophyll biosynthesis (Shalygo *et al.,* 1999). The heavy metals, including Co also alter the photosynthetic activity of the plants at multiple levels such as pigments, stomatal conductance, electron transport chain, enzymes and thylakoid membrane (Mysliwa-Kurdziel *et al.,* 2004).

In light of above, the present research was designed with an objective to investigate the effect of different levels of Co on the seed germination and antioxidant system in chickpea (*Cicer arientinum* L.) to workout the tolerable and toxic limits of the metal.

Materials and Methods

The seeds of chickpea (*Cicer arietinum* L.) cv. T-59, obtained from National Seed Corporation Ltd. New Delhi, India, were surface sterilized with 0.01% mercuric chloride solution followed by repeated washing with double distilled water. These seeds were subsequently soaked in water (control), 50, 100, 150 or 200 µM of Co in the form of cobaltous chloride for 12 hrs. The treated seeds were transferred to sterilized petriplates containing absorbent cotton, moistened with double distilled water. These petriplates were kept in an incubator run at a temperature of 25±2°C, in dark for 72 hrs (including the duration of the treatment). The relative water content (RWC), mean daily germination (MDG), final germination percentage (GP), the level of protein, activity of nitrate reductase (NR), lipid peroxidation and antioxidant enzymes (catalase, peroxidase and superoxide dismutase) were studied at 36 and 72 hrs after imbibition, as per Jones and Turner (1978), Gerson and Honma (1978) and Jaworski (1971).

The germinating seeds were homogenized in phosphate buffer (pH 7.0), and added with 0.2M potassium nitrate and 5% isopropanol solutions. This reaction mixture was incubated at 30±2°C for 2 hrs. Sulphanilamide (1%) and 0.02% NED-HCl solution were added to this mixture. The absorbance was read at 540 nm in a spectrophotometer.

Protein content : The method of Lowry *et al.* (1951) was followed to determine the quantity of protein in the samples. Proline content was determined following the method of Bates *et al.* (1973). The germinating seeds were homogenized with 3% aqueous sulphosalicyclic acid and centrifuged. To the supernatant, glacial acetic acid and acid ninhydrin solutions were added and this reaction mixture was heated in a water bath at 60°C for 1 hr and then cooled. Toluene was added to this reaction mixture and the colour of the toluene layer was read at 520 nm on a spectrophotometer.

Lipid peroxidation : Lipid peroxidation was estimated by measuring MDA content following the method of Hodges *et al.* (1999). 0.5 g of the leaf was homogenized in a mortar with 80% ethanol. The homogenate was centrifugted at 3000 g for 10 min at 4°C. The pellet was extracted twice with the same solvent. The supernatants were pooled and 1 ml of this sample was added to a test tube with an equal volume of the solution comprising 20% trichloroacetic acid, 0.01% butylated hydroxy toluene and 0.65% thioberbutyric acid. Samples were heated at 95°C for 25 min and cooled to room temperature. Absorbance of the samples was recorded at 440, 532 and 600 nm. The MDA content was expressed in n mol ml^{-1}.

Assay of antioxidant enzymes : The leaf tissue was homogenized in 50 mM phosphate buffer (pH 7.0) containing 1% (w/v) soluble polyvinylpyrrolidone. The homogenate was centrifuged at 15,000 rpm for 10 min at 5°C and the supernatant obtained was used as extract for estimating peroxidase, catalase and superoxide dismutase activities.

For estimating peroxidase activity (Chance and Maehly, 1956), 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 minutes, at the interval of 20 seconds, at 420 nm on a spectrophotometer. A control set was prepared by adding DDW instead of enzyme extract.

The reaction mixture for catalase consisted of phosphate buffer (pH 6.8), 0.1 M H_2O_2 and enzyme extract (1.0 cm^3). H_2SO_4 was added to the reaction mixture, after incubating it for 1 minutes at 25 °C, and was titrated against solution potassium permagnate solution (Chance and Maehly, 1956).

The activity of superoxide dismutase was measured by the method of Beauchamp and Fridovich (1971). A 3 ml of reaction mixture containing 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 2 mM riboflavin, 0.1 mM EDTA and 0.1 ml of enzyme extract was prepared. Riboflavin was added at last. This reaction mixture was exposed to low fluorescent light and decrease in the absorbance of the reaction mixture was read at 560 nm on a spectrophotometer. 50 % inhibition was considered as one enzyme unit.

Statistical analysis: The experiment was conducted with four independent replicates. The data were subjected to one way analysis of variance (ANOVA) and the significance of various treatments at 5% level and standard error was also calculated.

Results and Discussion

The relative water content (RWC) of the seeds increased as the germination progressed from 36 hr to 72 hr (Table 1). However, the seed soaking in Co concomitantly decreased the RWC, at both the samplings. Moreover, soaking the seeds in 150 and 200 µM Co concentration generated a comparable toxicity. However, the values for RWC after these treatments were significantly lower than control. The two lower concentrations (50 and 100 µM) were lesser toxic as compared to the two higher (150 and 200 µM) ones, but the response generated by these concentrations was also lower than that of control.

Mean daily germination exhibited a linear decrease in response to the treatments. However, an increase was noticed with the passage of time. The highest concentration (200 µM) proved most toxic and decreased MDG by 62 and 45%, compared to control, at 36 and 72 hr of sampling, respectively.

Treating seeds with Co significantly declined the per cent germination, which was proportionate to the concentration of metal in soaking medium, at both samplings (Table 1). Out of the

four concentrations tested, the highest one (200 µM) caused maximum inhibition in germination. It decreased the germination by 32 and 43%, compared to the control, at 36 and 72 hours of treatment, respectively. The lowest concentration (50 µM) showed a neglegible impact and germination in these seeds was comparable with that of control.

As seed germination progressed, the protein content and NR activity increased irrespective of the treatment (Table 2). The level of protein and enzyme activity in the seeds treated with Co was significantly lower than control. The highest concentration (200 µM) of Co was most inhibitory that decreased the protein content by 28 and 39% and enzyme activity by 24 and 22%, compared to control, at 36 and 72 hr of germination, respectively.

The proline content exhibited a linear increase as the seed germination progressed (Table 2). Treating seeds with Co also increased the proline content, which was proportionate to the concentration of metal. The highest concentration (200 µM) of metal caused maximum accumulation of proline, which was 69 and 60%, higher than control, at 36 and 72 hr of germination, respectively. Among different concentrations of Co, the seed soaked lowest concentration (50 µM) possessed the least proline, although the response was significantly higher than control.

The response of seeds to different concentrations of Co in terms of lipid peroxidation was similar to that of proline level, as evident from increased TBARS production (Table 2). In comparison to control, 200 µM Co increased the TBARS production by 85 and 78%, at 36 and 72 hrs of germination. However, the lower two (100 and 150 µM) concentrations generated a comparable effect, which was higher than that of 50 µM concentration, as well as control.

The activity of antioxidantive enzymes (catalase, peroxidase and superoxide dismutase) exhibited an increase with the passage of time (Table 2). However, the activity of these enzymes exhibited a decreaseing trend with increasing concentration of Co. The enzymes were severely affected 200 µM Co concentration. This concentration decreased at catalase activity by 37 and 36%; peroxidase by 41 and 45%; and superoxide dismutase by 28 and 36% as compared to control, at 36 and 72 hr of treatment, respectively. Other concentration of Co also declined of the enzyme activity significantly. However, the intensity was lesser than 200 µM Co.

The process of seed germination practically starts with the adsorption of water molecules by various hydrophilic groups (-NH_3, -OH and/or –COOH) of proteins and carbohydrates, located

Table 1 : Effect of cobalt on relative water content (%), germination percentage (GP), mean daily germination (MDG) (%), protein content (%) and NR activity (n mol NO_2 g^{-1} h^{-1} f.wt.) in the germinating seeds of *Cicer arietinum* at 36 and 72 hr of germination

Cobalt (µM)	RWC (%)		MDG (%)		GP (%)		Protein (%)		NR activity	
	36 hr	72 hr	36 hr	72 hr	36 hr	72 hr	36 hr	72 hr	36 hr	72 hr
Control	72 ± 4.3	86 ± 5.8	3.02 ± 0.74	3.40 ± 0.65	75.2 ± 6.8	90.3 ± 5.2	19.0 ± 1.5	22.5 ± 2.3	218 ± 9.2	260 ± 8.5
50	69 ± 5.3	82 ± 7.1	2.96 ± 0.55	3.12 ± 0.45	74.0 ± 5.4	91.3 ± 6.8	18.1 ± 1.9	21.2 ± 1.7	230 ± 11.5	282 ± 7.9
100	64 ± 7.1	74 ± 4.9	2.25 ± 0.47	2.73 ± 0.53	66.6 ± 7.2	85.5 ± 4.3	16.6 ± 1.3	17.8 ± 2.1	203 ± 8.3	246 ± 10.4
150	53 ± 6.5	61 ± 5.3	1.63 ± 0.62	2.17 ± 0.60	58.3 ± 6.1	73.6 ± 5.5	15.2 ± 1.8	16.6 ± 1.7	182 ± 7.4	219 ± 9.3
200	40 ± 3.7	46 ± 4.3	1.15 ± 0.42	1.85 ± 0.38	50.8 ± 4.9	59.1 ± 4.9	13.6 ± 1.5	14.4 ± 1.8	166 ± 8.8	202 ± 7.2
LSD at 5% level	5.2	6.5	0.46	0.31	4.7	3.7	1.30	1.45	13.6	16.4

Values are mean of four replicates ± SE

Table 2 : Effect of cobalt on lipid peroxidation (TBARS formed g^{-1} f.wt.), proline content (m mol g^{-1} f.wt.), catalase activity (m mol H_2O_2 decomposed g^{-1} f.wt.), peroxidase activity (units g^{-1} f.wt.) and superoxide dismutase activity (units g^{-1} f.wt.) in the germinating seeds of *Cicer arietinum* at 36 and 72 hr of germination

Cobalt (µM)	Proline content		Lipid peroxidation		Catalase activity		Peroxidase activity		SOD activity	
	36 hr	72 hr	36 hr	72 hr	36 hr	72 hr	36 hr	72 hr	36 hr	72 hr
0	9.39 ± 1.67	11.81 ± 1.97	26.0 ± 3.3	32.8 ± 3.9	163 ± 13.6	195 ± 13.0	17.3 ± 2.9	21.7 ± 3.4	138 ± 9.4	165 ± 8.6
50	10.95 ± 1.93	12.90 ± 2.18	28.3 ± 3.8	36.3 ± 4.3	158 ± 14.2	192 ± 16.4	15.9 ± 3.6	20.5 ± 2.5	127 ± 7.6	152 ± 6.2
100	12.10 ± 2.10	13.16 ± 2.06	34.4 ± 2.3	43.6 ± 3.3	136 ± 11.6	173 ± 13.8	13.8 ± 3.4	16.2 ± 3.2	116 ± 8.2	133 ± 7.5
150	14.47 ± 1.55	16.80 ± 2.88	40.3 ± 4.5	48.0 ± 3.8	113 ± 9.5	146 ± 9.7	12.6 ± 3.6	14.5 ± 2.2	105 ± 7.0	121 ± 6.8
200	15.88 ± 1.84	18.90 ± 2.25	48.1 ± 4.4	58.4 ± 3.5	102 ± 10.2	125 ± 11.0	10.2 ± 2.5	12.0 ± 2.7	86 ± 7.5	105 ± 65
LSD at 5% level	1.20	1.40	2.72	3.10	7.05	8.60	1.70	1.50	8.35	9.22

Values are mean of four replicates ± SE

in the seed coat, and form a hydrated shell around the macromolecules facilitating the existence imbibition force, against which water rushes into the seed resulting in its swelling. This step to a large extent is determined by water potential of the surrounding medium. In the present research, relative water content in the seeds of chickpea was significantly decreased by cobalt, in a dose dependent manner (Table 1). It was a consequence of the decrease in water potential caused by the presence of $CoCl_2$ salt. The next phase in the germination involves the activation and/or *de novo* synthesis of hydrolytic enzyme, particularly those involved in protein, carbohydrates, fats and lipid metabolism. These enzymes break down the complex reserves, which are subsequently mobilized to the growing axis. This phase is also accompanied with a drastic initial burst in respiration, together with the production of a large quantity of active oxygen species (AOS), such as, superoxide radicle ($O_2^{\cdot}$), hydroxyl radicle ($HO^{\cdot}$) and hydrogen peroxide (H_2O_2) (Bailey, 2004). These AOS are potentially toxic byproducts of respiratory electron transport chain and cause severe damage to the vital cellular components, such as proteins/enzymes, lipids, nucleic acids, plasma membrane and different metabolites (Helliwell and Gutterridge, 1999), mediated through the generation of oxidative stress. The advent of oxidative stress in our study was evident from increase in MDA content or lipid peroxidation, both in presence and absence of Co treatment. The heavy metals induced increase in lipid peroxidation, mediated through the generation of oxidative stresss is well documented (Schutzendubel and Poll, 2002, Bhardwaj *et al.*, 2009). Therefore, the seeds exposed to Co exhibited a significant increase in the level of MDA content (Table 2).

Production of AOS *per se*, by the germinating seeds in response to stress has been identified as cause of oxidative stress that might affect the germination process. However, in a natural routine seeds/plants are well equipped with enzymatic and non-enzymatic defence mechanisms to counter the toxicity of AOS, rather then to eliminate them completely (Bailly, 2004). The antioxidant enzymes and compounds have been widely considered as being of particular importance for the completion of germination. The activities of antioxidant enzymes catalase, peroxidase and superoxide dismutase were lower at early stage (36 hr) of germination than later stage (72 hr) (Table 2). Similar stimulation of antioxidant enzymes has been reported in *Arachis hypogea* (Jaleel et al., 2008) and *Vigna radiata* (Jaleel et al., 2009), exposed to cobalt, in a concentration dependent manner. Changes in AOS detoxifying enzymes (peroxidase and superoxide dismutase) are less documented than catalase, although there is a general trend for stimulation of these enzymes. However, a noteworthy observation recorded in the present study is that the activities of catalase, peroxidase and superoxide dismutase registered a concomitant decrease in response to Co treatment, which was reciprocal to that of lipid peroxidation. Therefore, it can be inferred that decline in the activities of these enzymes was mediated through an oxidative stress, evident from increased lipid peroxidation (Table 2). The toxicity of heavy metals is also attributed to their ability of binding to the enzymes, resulting in the alteration of their catalytic functions and inactivation (Ali *et al.,* 2008). The oxidative and/or metal stress induced inhibition was also observed by NR activity (Table 1).

However, contradictory to the response of enzymatic antioxidants to Co, the level of proline exhibited a concomitant increase as Co concentration increased (Table 2). The synthesis of proline involves enzymes Δ^1 pyrroline-5-carboxylate synthetase (Δ^1P5CS) and Δ^1pyrroline-5-carboxylate reductase ($\Delta^1$5CR). However, its subsequent degradation was mediated by proline dehydrogenase (ProDH). It is the proportion of these two set of enzymes, under stress, that increased proline content (Sumithra and Reddy, 2004). Kishore *et al.* (1995) noted over-expression of the gene/s coding Δ^1P5CS and repression of that responsible for ProDH in response to water stress in transgenic plants. The seeds exposed to cobalt, in the present study, possibly experienced water stress evident from decreased relative water content (Table 1), therefore, possessed more proline (Table 2). The increase in the proline pool is a general response of plants to various abiotic stresses, including that of heavy metals (Schat *et al.,* 1997). The mechanism and functional significance of proline accumulation in plants under heavy metal stress has been controversial. Most of the studies have demonstrated increased level of proline as a biochemical adaptation of the plants to maintain water balance and scavenge ROS generated through water stress caused by heavy metals (Schat *et al.,* 1997). However, Kastori *et al.* (1992) observed proline accumulation in metal exposed leaf discs and argued that this was due to metal uptake per se, rather than to water deficit stress. In the present research, increased level of proline could be a biochemical adaptation to scavenge the active oxygen species, which remained there in the cells as a consequence of the decline in the activity of the antioxidant enzymes.

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