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Alterations in the activities of antioxidative enzymes and increase in lipid peroxidation level in germinating mungbean seeds exposed to cobalt stress

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ABSTRACT

Mungbean (*Vigna radiata* (L.) Wilczek) seeds were subjected to germination in presence of $CoCl_2.6H_2O$ (0.5, 1.0 and 10.0 mM) for 48 h and different physiological parameters like soluble protein content, activities of antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) along with the lipid peroxidation level of the embryonic tissues were estimated in order to determined the effect of Co toxicity in germinating seeds. With increase in Co concentrations, the germination percentage decreased by 15% up to 1 mM which further reduced to 25% at 10 mM $CoCl_2.6H_2O$. Soluble protein content increased at 0.5 mM $CoCl_2.6H_2O$, but decreased further and at 10 mM concentration it was decreased by 53%. SOD activity was enhanced which indicated that there was protection against superoxide radical, but at the same time possibility of H_2O_2 accumulation as a dismutation product could not be ruled out. Since there was decrease in the activities of both catalase (CAT) and peroxidase (POX), protection against H_2O_2 was poor. Because of the alterations in the activities of these key enzymes, imposition of oxidative stress was presumed which was in fact observed in the form of increase in lipid peroxidation level in the tissues. The findings of the study indicate that Co at higher concentrations is toxic to the germinating seeds and imposition of oxidative stress might be one of the mechanisms behind such toxicity.

1. Introduction

Cobalt is a natural earth element that occurs in many different chemical forms in our environment. This element is present in trace amounts in soil, plants and also in our diets. In two valence states, i.e., Co II and Co III, it forms a number of organic and inorganic salts. Naturally cobalt occurs in association with other metals such as copper, nickel, manganese and arsenic. Cobalt and its salts are used to make superalloys, as paint drier, as a ground coat for porcelain enameling used on steel bathroom fixtures and large appliances; and also as an ingredient of coloured pigments. In environment, the natural sources of cobalt are soil, dust, seawater, forest fires and volcanic irruptions, burning of coal and petroleum products and also the industrial processes that use cobalt and its compounds in their manufacturing units. Cobaltite, smaltite and erythrite are the minerals in which cobalt occurs (Barceloux, 1999). The soil pollution with Co occurs mainly from mining and smelting activities, use of fertilizers and spreading of sewage sludge (Williams *et al*., 1985; Hamilton, 2000). Even though Co is essential as a micronutrient in trace amount by plants, higher concentrations have been reported to cause toxic effects (Chatterjee and Chatterjee, 2003; Osman *et al*., 2004). Studies on the adverse effects of Co on terrestrial ecosystems are a few. Some reports on the toxicity of Co on soil microbes and invertebrates are, however, available (Lock *et al*., 2004, 2006). Literature regarding toxic effects of Co on higher plants is limited. In this study, therefore, attempts have been made to assess the effect of Co on the antioxidative efficiency of germinating mungbean seeds under laboratory conditions.

2. Materials and methods

Fresh mungbean (*Vigna radiata* (L.) Wilczek) seeds were collected locally and healthy seeds for uniformity of

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size were selected. The seeds were then surface sterilized with freshly prepared 3% filtered solution of bleaching powder (calcium oxychloride, $CaOCl_2$) for 30 min followed by several washings with distilled water for another 30 min. Then the seeds were spreaded over filter paper moistened with 10 ml solutions of different concentrations of $CoCl_2.6H_2O$ (0.5, 1.0 and 10.0 mM) in separate Petri dishes. 10 ml of distilled water was taken in another Petri dish as control and 20 seeds were taken in each Petri dish. The seeds were allowed to germinate in dark at 30 ± 2^{0}C for 48 h and after that germinated seeds were collected for analytical studies.

After 48 h of germination, the number of seeds germinated in each case was counted. The embryonic tissues were collected separately (discarding the endosperm portions) and homogenized with sodium phosphate extraction buffer (0.0.5 M) in a mortar and pestle kept in an ice-bath. The pH of buffer was 7.4 for superoxide dismutase (SOD) and 7.5 for catalase (CAT) and peroxidase (POX). The homogenates were centrifuged in a cooling centrifuge (17000*g*) for 10 min at - 4^{0}C and supernatants were collected for assay of enzyme activities after suitable dilutions. Superoxide dismutase (EC 1.15.1.1) activity was assayed following the method of Das *et al*. (2000) and in this method inhibition of superoxide driven nitrite formation from hydroxylamine hydrochloride by SOD was measured spectrophotometrically at 543 nm. The activity was calculated by deducting one from the ratio of absorbance of the control (without enzyme) and sample (with enzyme). The SOD activity was expressed in unit, defined as the amount that inhibits the superoxide driven nitrite formation from hydroxylamine hydrochloride by 50% under the assay conditions. Catalase (EC 1.11.1.6) activity was measured following the method of Aebi (1983) taking H_2O_2 as the substrate and the rate of decreasing concentration of H_2O_2 due to the enzyme was measured spectrophotometrically at 240 nm. The activity (katal) was calculated using the extinction coefficient of 40.0/mM. cm for H_2O_2 at 240 nm. Peroxidase (EC 1.11.1.7) activity was assayed taking H_2O_2 and guaiacol as substrate and reduced co-substrate respectively following the method of Kar and Feierabend (1984). The rate of increase in the colour intensity in reaction mixture due to formation of tetraguaiacol was recorded spectrophotometrically at 470 nm and the activity (katal) was calculated using the extinction coefficient of 26.6/mM. cm due to tetraguaiacol formation.

A portion of the enzyme supernatant, for each sample, was mixed with equal volume of 20% (w/v) trichloroacetic acid (TCA) and kept in refrigerator for over night in order to facilitate the precipitation of buffer soluble protein. The precipitates were washed subsequently with 10% TCA, absolute alcohol, alcohol and chloroform (in a proportion of 3:1), alcohol and ether (in a proportion of 3:1) and finally with ether. After that, the pellets were air dried and re-suspended with 0.3 N NaOH solutions for 16 h at 37^{o}C. Then the samples were centrifuged and supernatants were collected as soluble protein extract. The protein content was estimated following the method of Lowry *et al.* (1951). Soluble protein content of the tissue was calculated from a standard curve drawn with bovine serum albumin as protein.

For determination of lipid peroxidation in the tissues malondialdehyde (MDA) was estimated as thiobarbituric-acid-reactive material from tissue extracts. The extraction was done in 5% (w/v) TCA and MDA content was estimated following the method of Heath and Packer (1968). During this estimation the unspecific turbidity in the reaction mixtures was corrected by subtracting the absorbance at 600 nm, and for absorbance at 532 nm originating from extract after incubation without thiobarbituric acid.

All the experiments were performed at least for three times with three replicates in each time. The mean values are presented and the standard deviations are also indicated.

3. Results and discussion

It has been reported in many studies that the supra-normal doses of different heavy metals cause relatively high toxicity, which are mostly reflected in terms of growth inhibition of plants accompanied by chlorosis of young leaves and other disorders including alterations in the antioxidative efficiency (Daniels *et al*., 1972; Kaer *et al*., 1998; Dey *et al*., 2007; 2009 a, b). In cauliflower it was reported by Chatterjee and Chatterjee (2000) that the activities of several enzymes including that of iron containing enzymes are disturbed by excessive amounts of heavy metals like Co, Cr and Cu. Among these metals Co has been found to readily translocated from old to new tissues (Handrek and Riceman, 1969). Co is usually translocated through xylem sap as divalent cation in plants (Tiffin, 1967).

Germination is an important phase in the growth and development of plant life during which upon absorption of water the tender embryonic tissues in the forms of radicle and plumule emerge out of the seed by rupturing the seed coat. Exposure of the embryonic tissues to any type of stress is expected to have adverse effect not only to the growing seedlings but also to the plants towards the later stages of growth and development. In this study it was found that with increase in Co in the medium, there was decrease in germination percentage and at highest concentration of the metal (i.e., 10 mM $CoCl_2$), 25% decrease

in comparison to the control seeds was recorded (Table 1). Soluble protein content of the embryonic tissues also decreased by 17% and 53% in the tissues exposed to 1.0 mM and 10.0 mM $CoCl_2$ respectively (Table 1), but at 0.5 mM $CoCl_2$, there was about 7.5% increase in comparison to control tissues. This shows that up to 0.5 mM concentration, probably Co was beneficial for the tissues as a micronutrient and beyond that it was toxic. The decrease in the soluble protein content is an indirect indication of the alteration in the activities of enzymes due to Co toxicity.

The antioxidative enzymes play a very important role in preventing the build up of reactive oxygen species (ROS) inside the cell. It has been reported by many workers that the toxicity due to exposure to different heavy metals like Cd (Schutzendubel *et al*., 2001), Pb (Verma and Dubey, 2003), Cd and Pb (Dey *et al*., 2007), Al (Cakmak and Horst, 1991), Cu (Srivastava *et al*., 2006; Dey *et al*., 2009 b) and Cr (Dey *et al.,* 2009 a) have altered the activities of antioxidative enzymes and have consequently imposed oxidative stress in the plants. In this study it was found that SOD activity increased in the embryonic tissues up to 1.0 mM $CoCl_2$ which further declined at 10 mM $CoCl_2$ but it was more than that of the control tissues (Fig. 1). The SOD catalyses the dismutation of superoxide radicals to hydrogen peroxide and oxygen and thus reduces the build up of superoxide radicals in the cell (Halliwell and Gutteridge, 2007). But at the same time, the increased SOD activity also increases the cellular levels of toxic H_2O_2 and therefore, decomposition of H_2O_2 is very vital. Thus, in this study even though protection against superoxide radical was there with increased Co concentration in the medium, but the possibility of increase in H_2O_2 concentration could not be ruled out.

Catalase is the principal enzyme responsible for decomposition of H_2O_2 to H_2O and O_2 and thereby reduces its toxicity (Elstner, 1982; Halliwell and Gutteridge, 2007). Peroxidases are also the antioxidative enzymes that decompose H_2O_2 with co-oxidation of reduced co-substrates (Elstner, 1982). Here in this study, the activities of these two enzymes have been found to reduce with increase in the Co concentration in the medium (Fig. 1). This indicated that the protection against H_2O_2 was poor and there was possibility of build up of H_2O_2 inside the cell. Hydroxyl radicals (·OH) are known to be formed from H_2O_2 in aerobic cells in presence of transition metal ions (Halliwell and Gutteridge, 2007). Further, the elevated steady state levels of H_2O_2 and $O_2^{\cdot}$ can react in presence of transition metal ions to produce hydroxyl radical (·OH), via Haber-Weiss reaction (Elstner, 1982). The ·OH is known to be the most potential toxic species of oxygen and the unsaturated fatty acid components of membrane lipids are highly susceptible to ·OH attack and are peroxidized in presence of it. Therefore, increased lipid peroxidation is considered as a good indicator of prevalence of free radical mediated reactions in aerobic organisms (Kappus, 1985). In this study, the increase in the MDA content in the germinating embryonic tissues in presence of Co (Table 1) indicated the prevalence of oxidative stress in the tissues. Thus, imposition of oxidative stress might be one of the mechanisms behind Co induced toxicity in the germinating seeds.

Cobalt treatment in the soil at low concentration (i.e.,

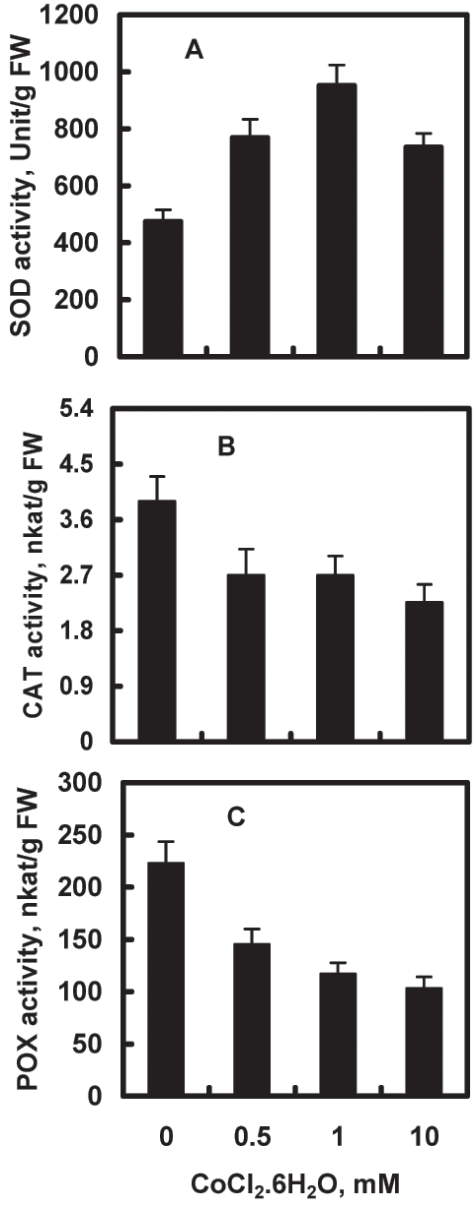

Fig. 1 Changes in the activities of superoxide dismutase (SOD) (A), catalase (CAT) (B) and peroxidase (POX) (C) in embryonic tissues of mungbean seeds germinated in presence of $CoCl_2.6H_2O$ for 48 h.

Table 1
Changes in the germination percentage; soluble protein content and lipid peroxidation level of embryonic tissues of mungbean seeds exposed to cobalt chloride for 48 h

$CoCl_2.6H_2O$ (mM)	Germination (%)	Soluble protein (mg/g FW)	MDA (nmol/g FW)
0	100	17.5 ± 1.2	25 ± 2.4
0.5	85 ± 4	18.8 ± 1.6	33 ± 3.7
1.0	85 ± 3	14.6 ± 1.1	44 ± 4.5
10.0	75 ± 3	8.2 ± 0.9	56 ± 5.2

50 mg/kg soil) is reported to have beneficial effects on the growth of soybean plants (Jayakumar and Abdul Jaleel, 2009). But higher concentrations reduced the growth. According to Stiborova *et al.* (1988), the lower concentration of Co increases the water and nutrient absorption efficiency of roots and thereby enhances the plant growth. In tomato seedlings, treated with 0.5 mM Co, Gopal *et al.* (2003) have observed various morphological as well as physiological anomalies. Along with other physiological anomalies they also found the disturbances in plant phosphorus and sulfur content due to excess Co in the seedlings which affected the carbohydrate and nitrogen metabolisms and that was the main reason behind depressed growth and lowered biomass. In cauliflower, excess of Co was found to inhibit the translocation of nutrients from roots to upper parts as a result there was reduction in the growth (Chatterjee and Chatterjee, 2000). All these findings suggest that Co at higher concentration is toxic to plants which manifest in the forms of different morpho-physiological anomalies. The results of this study suggest that during germination stage, excess of Co can alter the activities of antioxidative enzyme; increase lipid peroxidation level and consequently imposes oxidative stress in the germinating embryonic tissues. Probably imposition of oxidative stress might be one of the mechanisms behind Co induced toxicity in plants. Since during germination stage excess of Co has been found to have toxic effects, prolonged exposure of tender embryonic tissues to Co would definitely pose a threat for the developing seedlings. In fact, Co has also been reported to have toxic effects in different seedlings, as have already been outlined above. Therefore, remediation of soil contaminated with excess of Co is highly essential before sowing seeds or for other plantation programme.

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