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Antioxidant potentials protect *Vigna radiata* (L.) Wilczek plants from soil cobalt stress and improve growth and pigment composition

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Abstract

The experiments were conducted in earthen pots lined with polythene sheet to find out the effect of different concentrations of cobalt (0, 50, 100, 150, 200 and 250 mg/kg soil) on various morphological parameters, photosynthetic pigment contents and antioxidant enzyme activities on greengram (*Vigna radiata* (L.) Wilczek). Plants were watered to field capacity daily. Plants were thinned to a maximum of five per pot. The data for various morphological parameters such as, root and shoot length, number of nodules, dry weight of root and shoot and photosynthetic pigments such as chlorophyll 'a', chlorophyll 'b', total chlorophyll and carotenoids content were collected on 30 days after sowing (DAS). Antioxidant enzymes like catalase, peroxidase and polyphenol oxidase activities were analysed from both control and treated plants. All the growth parameters and pigment contents increased at 50 mg/kg cobalt level in the soil, when compared with control. Further increase in cobalt level (100-250 mg/kg) in the soil had a negative impact upon all studied parameters. From these results it is clear that Antioxidant potentials acts as a protective mechanism in *Vigna radiata* under soil cobalt stress.

Keywords: antioxidant potentials; cobalt; morphological parameters; photosynthetic pigment; greengram; *Vigna radiata*

Introduction

Heavy metal pollution is one of the most serious environmental problems which has been a subject of extensive research in recant years (Jayakumar et al., 2007). Heavy metals are dominantly found in almost all kinds of industrial and sewage wastes (Jaleel et al., 2009a). These metals in their normal oxidation states are generally found in sewage and industrial wastes. Large areas of land are contaminated with heavy metals due to natural process, particularly lithogenic and pedogenic, as well as anthropogenic factors such as industrial activity, mining, sewage, traffic, etc. The concentration of heavy metals in air, water and soil leads to many hazardous effects to living organisms. Excessive metal concentrations in contaminated soils can result in decreased soil microbial activity and soil fertility and yield losses (McGrath et al., 1995). The heavy metals can create a major ecological crisis since they are non-degradable and often accumulate by plant parts, biologically magnified through trophic levels and causing a deleterious biological effect. As a response to the toxic action of metals various protective mechanisms have been elaborated in plants (Kleizaite et al., 2004).

Cobalt (Co) as a trace element can be a contaminant in soils due to agricultural additives or metal refineries (Bakkaus et al., 2005). Certain plant species have the ability to extract metals (such as Co) from soils, thus, cleaning the environment. Co is known to cause irreversible damage to a number of vital metabolic constituents and plant cell and cell membrane. While it has been known for many years that Co is an essential element for humans, animals and prokaryotes, a physiological function for this element in higher plants has not been identified. The Co-containing vitamin B_{12} does not occur in plants. Trace elements are necessary for the normal metabolic functions of the plant, but at higher concentrations, these metals are toxic and may severely interfere with physiological and biochemical functions (El-Sheekh et al., 2003; Parmer and Chanda, 2005; Jayakumar and Vijayarengan, 2006).

In abiotic stress, metal response will results in the production of reactive oxygen species (ROS) which leads to the activation of defense mechanisms in terms of antioxidant enzymes. Generation of ROS such as superoxide, H_2O_2 and hydroxyl molecules cause rapid cell damage by triggering off a chain reaction (Imlay, 2003; Jaleel et al., 2006, 2007a). Plants under stress produce some defence mechanisms to protect themselves from the harmful effect of oxidative stress. ROS scavenging is one among the common defense response against abiotic stresses (Vranova et al., 2002; Jaleel et al., 2007b). ROS scavenging depends on the detoxification mechanism provided by an integrated system of non-enzymatic reduced molecules like ascorbate and glutathione and enzymatic antioxidants (Jaleel et al., 2007c). The major ROS scavenging activities includes complex non-enzymatic (ascorbate, glutathione, α-tocopherol) and enzymatic antioxidants like catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), superoxide dismutase (SOD), polyphenol oxidase (PPO), peroxidase (POX) etc. (Jaleel et al., 2007d). The pathways include the water-water cycle in chloroplasts and the ascorbate-glutathione cycle (Asada, 1999). Antioxidant mechanisms may provide a strategy to enhance metal tolerance in plants.

The present investigation was executed with an objective to study the effects of Co stress on morphological parameters and pigment contents of greengram with specific emphasize on antioxidant enzymes activities which are the defense mechanism to any type of abiotic stress.

Materials and methods

Seed collection and cultivation

Seeds of greengram (*Vigna radiata* (L.) Wilczek cultivar. ADT-5) were obtained from Tamil Nadu Rice Research Institute, Tamil Nadu Agricultural University, Aduthurai. The seeds with uniform size colour and weight were chosen for the present study and sown in pots containing 3kg air dried soil each. The inner surface of pots was lined with polythene sheet. The experiment was conducted in completely randomized block design (CRBD) method with 5 replicates in pots. Plants were grown in untreated soil (control) and in soil to which cobalt had been applied (50, 100, 150, 200 and 250 mg/kg soil). The cobalt as finely powdered cobalt chloride ($CoCl_2$) was applied to the surface soil and thoroughly mixed with soil. Ten seeds were sown in each pot. All the pots were watered to field capacity daily. Plants were thinned to a maximum of five per pot, after a week of germination.

Morphological parameters

Morphological parameters like shoot and root length, number of nodules and dry weight were measured in the samples. The total leaf area was calculated with LICOR Photoelectric Area Meter (Model LI-3100, Lincoln, USA).

Pigment analysis

Chlorophyll and carotenoid were extracted from the leaves and estimated by the method of Arnon (1949).

Extraction

Five hundred milligrams of fresh leaf material was ground with 10 ml of 80 per cent acetone at 4°C and centrifuged at 2500xg for 10 minutes at 4°C. This procedure was repeated until the residue became colourless. The extract was transferred to a graduated tube and made up to 10 ml with 80 per cent acetone and assayed immediately.

Estimation

Three milliliters aliquots of the extract were transferred to a cuvette and the absorbance was read at 645, 663 and 480 nm with a spectrophotometer (U-2001-Hitachi) against 80 per cent acetone as blank. Chlorophyll content was calculated using the formula of Arnon.

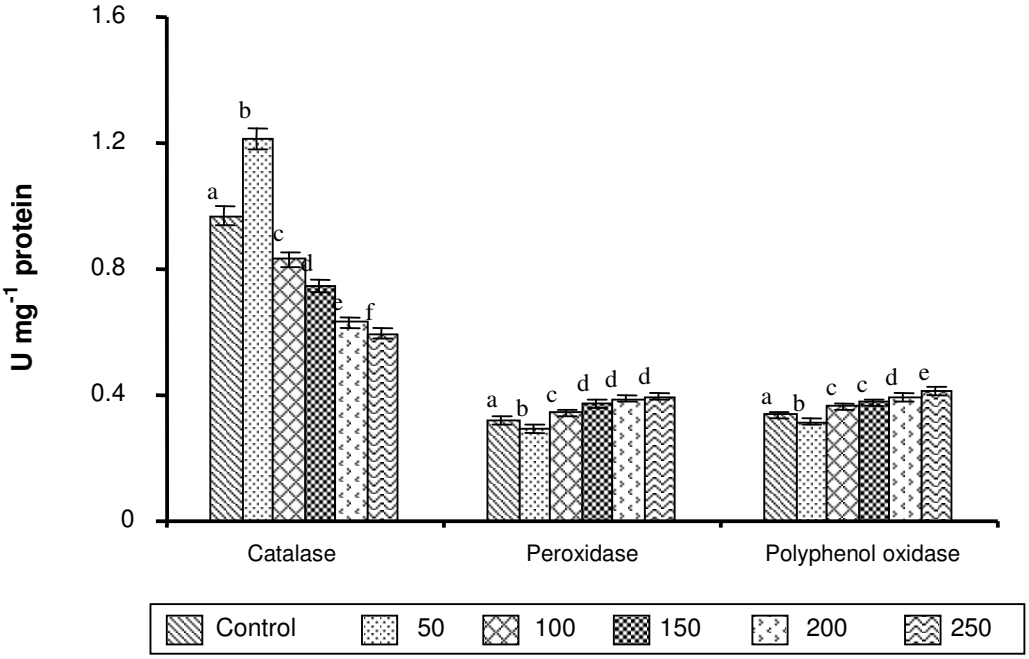

Fig1. Cobalt induced changes in antioxidant enzyme activities of *Vigna radiata*. Values are given as mean ± SD of six experiments in each group. Bar values are not sharing a common superscript (a,b,c,d,e,f) differ significantly at $P \leq 0.05$ (DMRT).

Total chlorophyll (mg/ml)=(0.0202) × (A.645)+ (0.00802) × (A.663)
Chlorophyll 'a' (mg/ml)=(0.0127) × (A.663)– (0.00269) × (A.645)
Chlorophyll 'b' (mg/ml) =(0.0229) × (A.645)– (0.00468) × (A.663)
and expressed in milligram per gram fresh weight.

Carotenoid content was estimated using the formula of Kirk and Allen (1965) and expressed in milligrams per gram fresh weight.
Carotenoid = A.480 + (0.114 × A.663 – 0.638 × A.645).

Antioxidant enzymes

Catalase (CAT) (EC 1.11.1.6) activity was measured according the method of Chandlee and Scandalios (1984) with small modification. 0.5 g of frozen plant material was homogenized in a prechilled pestle and mortar with 5 ml of ice cold 50 mM sodium phosphate buffer (pH 7.5) containing 1 mM phenyl methyl sulfonyl fluoride (PMSF). The extract was centrifuged at 4 °C for 20 min at 12,500 xg. The supernatant was used for enzyme assay. The assay mixture contained 2.6 mL of 50 mM potassium phosphate buffer (pH 7.0), 400 µL of 15 mM H_2O_2 and 40 µL of enzyme extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm.

Peroxidase (POX; EC 1.11.1.7) was assayed by the method of Kumar and Khan (1982). Assay mixture of POX contained 2 mL of 0.1 M phosphate buffer (pH 6.8), 1 mL of 0.01 M pyrogallol, 1 mL of 0.005 M H_2O_2 and 0.5 mL of enzyme extract. The solution was incubated for 5 min at 25 °C after which the reaction was terminated by adding 1 mL of 2.5 N H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 2.5 N H_2SO_4 at zero time. The activity was expressed in unit mg^{-1} protein. One unit (U) is defined as the change in the absorbance by 0.1 min^{-1} mg^{-1} protein.

Polyphenol oxidase (PPO; EC 1.10.3.1) activity was assayed by the method of Kumar and Khan (1982). Assay mixture for PPO contained 2 mL of 0.1 M phosphate buffer (pH 6.0), 1 mL of 0.1 M catechol and 0.5 mL of enzyme extract. This was incubated for 5 min at 25 °C, after which the reaction was stopped by adding 1 mL of 2.5 N H_2SO_4. The absorbancy of the benzoquinone

Table 1. Effect of cobalt on growth parameters of greengram (30th day)

Cobalt added in the soil (mg/kg)	Root length	Shoot length	Number of nodules	Root dry weight	Shoot dry weight
Control	41.34 [a]	48.61 [a]	39.81 [a]	0.312 [a]	0.819 [a]
50	44.09 [b]	52.18 [b]	43.18 [b]	0.386 [a]	0.897 [a]
	(+6.652)	(+7.344)	(+8.465)	(+23.71)	(+9.523)
100	39.68 [c]	43.54 [c]	37.22 [c]	0.274 [b]	0.761 [b]
	(–4.015)	(–10.42)	(–6.505)	(–12.17)	(–7.081)
150	32.21 [d]	39.86 [d]	31.14 [d]	0.234 [c]	0.673 [c]
	(–22.08)	(–18.00)	(–21.77)	(–25.00)	(–17.82)
200	26.17 [e]	33.47 [e]	24.62 [e]	0.198 [d]	0.518 [d]
	(–36.69)	(–31.14)	(–38.15)	(–36.53)	(–36.75)
250	19.74 [f]	28.94 [f]	17.31 [f]	0.163 [e]	0.434 [e]
	(–52.24)	(–40.46)	(–56.51)	(–47.75)	(–47.00)

(Percent over control values are given in parenthesis)

Values are given as mean ± SD of six experiments in each group. Values that are not sharing a common superscript (a, b, c, d, e, f) differ significantly at $P \leq 0.05$ (DMRT).

formed was read at 495 nm. To the blank 2.5 N H_2SO_4 was added of the zero time of the same assay mixture. PPO activity is expressed in U mg^{-1} protein (U = Change in 0.1 absorbance min^{-1} mg^{-1} protein). The enzyme protein was estimated by the method of Bradford (1976) for expressing all the enzyme activities.

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). The values are mean ± SD for six samples in each group. P values ≤ 0.05 were considered as significant.

Results and discussion

Growth and dry matter yield

The root and shoot length of *Vigna radiata* plants at 30 DAS under Co treatment is represented in Table 1. The root and shoot length of *Vigna radiata* increased with age of the plants and decreased (except 50 mg kg^{-1}) with increase in the concentration of Co in the soil. The highest root and shoot length of *Vigna radiata* was observed at 50 mg kg^{-1} on 30 DAS and lowest root and shoot length was observed at 250 mg kg^{-1} on 30 DAS.

The root and shoot dry weight of *Vigna radiata* plants raised in various levels of Co is furnished in Table 1. When compared to the control 50 mg kg^{-1} Cobalt level in the soil increased the dry weight of root and shoots and decreased the same at high levels (100-250 mg kg^{-1}). Root and shoot length of *Vigna radiata* plants decreased with an increase in Co level in the soil. Root and shoot length were found to be higher at 50 mg kg^{-1}. Similar decrease in plant height was reported previously (Jayakumar and Vijayarengan, 2006). Co at high levels may inhibit the root and shoot growth directly by inhibition of cell division or cell elongation or combination of both, resulting in the limited exploration of the soil volume for uptake and translocation of nutrients and water and induced mineral deficiency (Hemantaranjan et al., 2000).

The dry mater production of root and shoot was highest at 50 mg kg^{-1} Co level. But it showed a gradual decline from 100 mg kg^{-1} level onwards. There is large number of reports that the heavy metal increased the dry matter yield of various plants at lower levels (Jayakumar and Vijayarengan, 2006). The reduction in dry matter yield of plants at higher concentration of heavy metals was also observed previously in various plants (Vijayarengan, 2004).

Photosynthetic pigments

Photosynthetic pigments such as chlorophyll 'a', chlorophyll 'b', total chlorophyll and carotenoid content of *Vigna radiata* leaves increased at lower concentration (50 mg kg^{-1}). Photosynthetic pigments such as chlorophyll 'a', chlorophyll 'b', total chlorophyll and carotenoid contents of groundnut decreased with increasing Co level in the soil. Similar changes in the content by various metal treatments were recorded (Schlegel

Table 2. Effect of cobalt on chlorophyll and carotenoid content (mg g^{-1} fresh weight) of green gram (30^{th} day)

Cobalt added in the soil (mg/kg)	Chlorophyll 'a'	Chlorophyll 'b'	Total Chlorophyll	Carotenoid
Control	0.719 [a]	1.143 [a]	1.862 [a]	0.093 [a]
50	0.876 [b]	1.964 [b]	2.840 [b]	0.132 [b]
	(+21.83)	(+71.82)	(+52.52)	(+41.93)
100	0.648 [c]	1.012 [c]	1.660 [c]	0.086 [c]
	(–9.874)	(–11.46)	(–10.84)	(–7.526)
150	0.517 [d]	0.785 [d]	1.392 [d]	0.064 [d]
	(–28.09)	(–31.32)	(–25.24)	(–31.18)
200	0.426 [e]	0.673 [e]	1.099 [e]	0.042 [e]
	(–40.75)	(–41.11)	(–40.97)	(–54.83)
250	0.302 [f]	0.397 [f]	0.699 [f]	0.037 [e]
	(–57.99)	(–65.26)	(–62.45)	(–60.21)

(Percent over control values are given in parenthesis)

Values are given as mean ± SD of six experiments in each group. Values, that are not sharing a common superscript (a,b,c,d,e,f) differ significantly at $P \leq 0.05$ (DMRT).

et al., 1987). The increased chlorophyll content at lower level of Co was obviously due to better growth.

The excess Co treatment brought about by a marked depression in photosynthetic pigment in plants. It might be due to excess supply of Co resulting in interference with the synthesis of chlorophyll. The formation of chlorophyll pigment depends on the adequate supply of iron. Granick (1951) has suggested protoporphyrin is a precursor for chlorophyll synthesis. The excess supply of Co seems to prevent the incorporation of iron in protoporphyrin molecule resulting in the reduction of chlorophyll pigment. This was strengthened by the fact that excessive amounts of a range of heavy metals such as copper (Mocquot et al., 1996), cobalt (El-Sheekh et al., 2003) induced chlorosis in plants which were usually similar to the chlorosis of iron deficiency. Impaired chlorophyll development by heavy metals may be due to the interference to protein, the treatments presumably blocked the synthesis and activities of enzyme proteins responsible for chlorophyll biosynthesis.

Antioxidant enzymes

The leaf CAT activity was high in 50 mg kg^{-1} and it was low in 250 mg kg^{-1}. The increase in metal concentration decreased the CAT activity. POX and PPO activities in leaves were high in 250 mg kg^{-1} and it was low in 50 mg kg^{-1} (Fig. 1). These enzymatic studies showed that the increase in metal concentration there was an increase in POX and PPO activities.

CAT activity decreased with increasing concentration of Co (100-250 mg kg^{-1}) than the control and low level of Co (50 mg kg^{-1}) treated *Vigna radiata* plants. POX and PPO activities increased (except 50 mg kg^{1}) with an increase in Co level in the soil. This can be compared with earlier reports such as Savour et al. (1999) and Chen et al. (2001).

To be able to endure oxidative damage under conditions which favours increased oxidative stress such as high/low temperatures, water deficit, and salinity etc., plants must possess efficient antioxidant system (Jaleel et al., 2008a, 2009b). Plants posses antioxidant systems in the form of enzymes such as SOD, APX, CAT and metabolites viz., ascorbic acid, glutathione, α-tocopherol, carotenoid, flavonoids, etc. (Jaleel et al., 2008b). These antioxidant enzymes and metabolites are reported to increase under various environmental stresses (Jaleel et al., 2008c) as well as comparatively higher activity has been reported in fungicide, triadimefon (Jaleel et al., 2008d) and salt treatments (Jaleel et al., 2008e) in medicinal plants, suggesting that higher antioxidant enzymes activity have a role in imparting tolerance against any type of environmental stresses.

Conclusion

Co treatment at all levels tested (except 50 mg kg^{-1}) decreased the various growth parameter such as root ad shoot length, number of nodules, total leaf area and dry weight of root and shoot; biochemical (pigment, sugar, starch, amino acid and protein) contents of leaves; antioxidant enzyme (CAT) activity of *Vigna*

radiata plants. However the antioxidant enzymes (POX and PPO) increased with an increase in Co level in the soil. From the present investigation it can be concluded that the 50 mg kg^1 level of Co in the soil is beneficial for the growth of *Vigna radiata* plants.

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