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Full Length Research Paper

Effects of Cobalt Chloride Stress on Physiological, Biochemical, and Enzymatic Parameters and Protein Profile in *Cyamopsis tetragonoloba*

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We investigated the changes in the morphology, biochemical, antioxidant enzyme activity and protein profiles in *Cyamopsis tetragonoliba* (L). The crop was treated with different concentrations of $CoCl_2$ (25, 50, 75, 100, 125 μM^{-1}) and the changes in growth, protectively induced oxidative stress in relation to metal stress after 14 days old plant were examined. In this study, the lower concentration of 25 µM cobalt chloride has increased the plant productivity than the other concentrations tested. Chlorophyll and carotenoid pigments decreased significantly in the stressed leaves for their adaptation in stressed condition. The two isozymes CAT 1 and CAT 2 activity were detected from the extract of treated and stress leaves were subjected to polyacrylamide gel electrophoresis. Most prominent peroxidase enzymes were induced by accumulation $CoCl_2$ and possibly resulting in improved effectiveness of the enzyme at high concentration of stressed leaves. The protein profile of cluster bean (*Cyamopsis tetragonoliba* L.) strongly influenced by severe heavy metal stress (100 µM and 125 µM). It was analyzed through SDS-PAGE and found four new polypeptides, hence it was concluded that more number of new proteins (190 kDa, 54 kDa, 38 kDa and 33 kDa) were synthesized in stress treated plants and it was highly intensive and probably constitutes stress tolerant proteins.

Keywords: Biomass, protein, antioxidants, cluster bean, $CoCl_2$.

INTRODUCTION

Cluster bean (Cyamopsis tetragonoliba L.) Taub is the most important food legume. It increases the amount of nitrogen in the soil, used as green manure. It grows in a wide range of rain fall areas, from 400 mm to 2400 mm and grows best in highly humid conditions. The cluster bean is a bushy branching annual with a lateral root system and its immature pods and seeds; gum extracted from seeds is commercially important thickening agent for food industry. Globally, the production is around 7.5 to 10 lakh tons every year (Hymowitz and Matlock 1963). India is the leading producer of cluster bean and it produces 80% of the total production in the world. They are mainly affected by ultimate disposal of treated and untreated waste containing toxic metals as well as metal chelates from tannery, steel plants, battery industries and thermal power plants. The indiscriminate use of heavy metals is highly toxic environmental pollutants (Ammann (2002)).

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Table 1. Effect of cobalt toxicity on morphological and biometric parameters of *C.tetragonoloba* L on two weeks old plant.

S.No	Cobalt concentration in soil (µM)	Shoot length (cm/plant)	Root length (cm/plant)	Total leaf area (cm^2)	Leaf fresh weight (g/plant)	Leaf dry Weight (g/plant)
1	Control	17.62 ± 0.16**	7.32 ± 0.14 *	12.31 ± 0.31 **	0.35± 0.19 ***	0.05 ± 0.01 **
2	25µM	18.66 ± 0.45*	8.64 ± 0.38 *	14.62 ± 0.29 ***	0.67 ± 0.27 **	0.06 ± 0.02 *
3	50µM	14.93 ± 0.61*	6.96 ± 0.21 **	13.08 ± 0.12 **	0.68 ± 0.25 **	0.08 ± 0.02 *
4	75µM	12.08 ± 0.23 **	6.06 ± 0.18 **	11.35 ± 0.33 *	0.35 ± 0.11 ***	0.04 ± 0.01 *
5	100µM	10.91 ± 0.41 **	5.61 ± 0.10 **	10.64 ± 0.15 **	0.32 ± 0.18 *	0.03 ± 0.001 **
6	125µM	8.98 ± 0.39 **	4.06 ± 0.24 *	7.05 ± 0.34 **	0.05 ± 0.03 *	0.02 ± 0.002 **

Values represent the mean SE. Means ± standard error. Means with the same letter within a column are not significantly different, according to Duncan's multiple range tests: *P<0.05; **P < 0.01; *** P< 0.001.

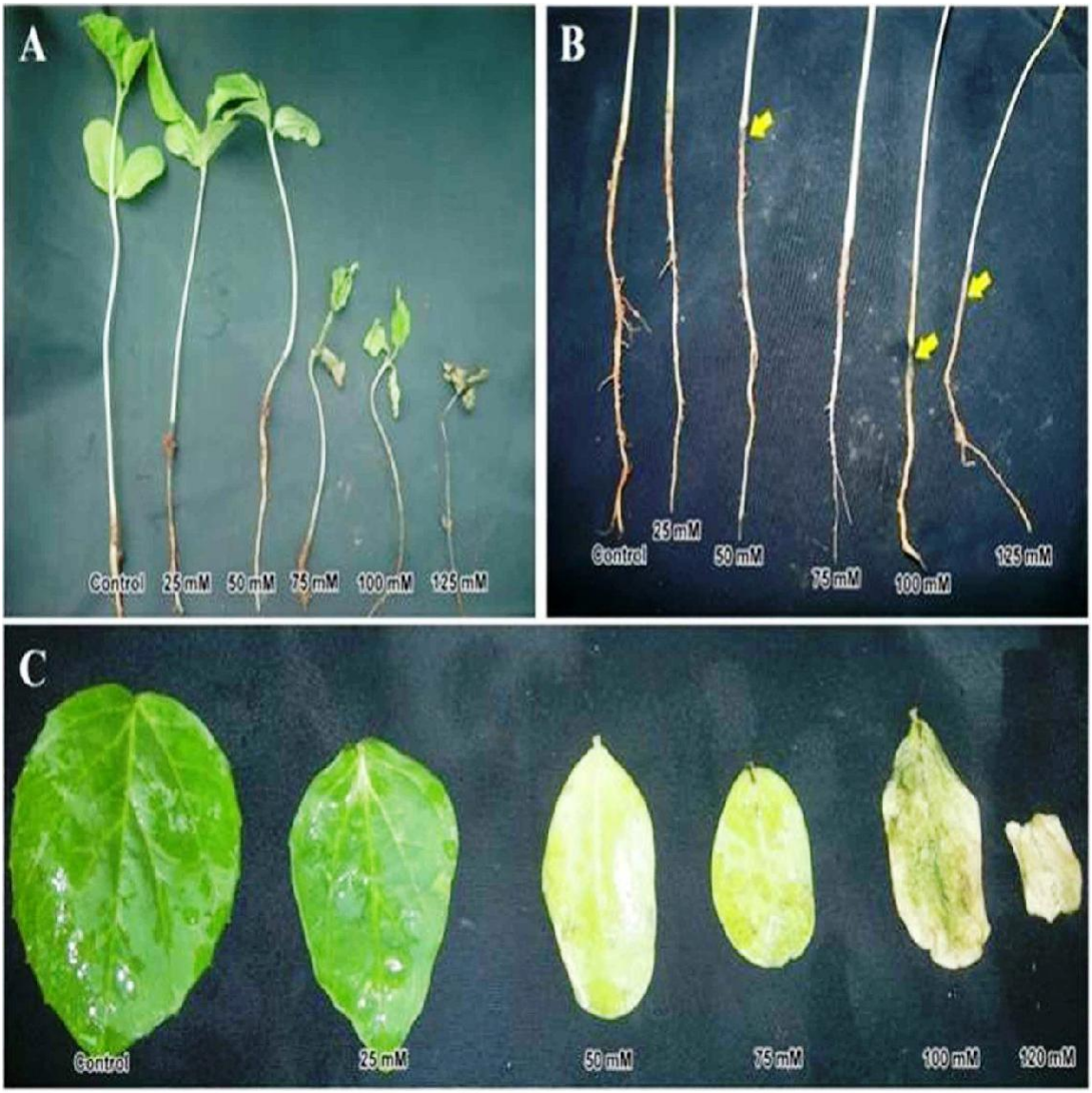

Figure 1. Morphological changes of *Cyamopsis tetragonoloba* L. grown under different concentration of $CoCl_2$. (A) Uprooted view, (B) Blackening of root under cobalt stress (Indicated by arrow). (C) Leaf chlorosis and necrosis at different concentration of $CoCl_2$.

Table 2. Effect of cobalt toxicity on photosynthetic pigments content of *Cyamopsis tetragonoloba* L. on two weeks old plant.

S.No	Concentration of cobalt in soil (µM)	Chlorophyll 'a' content (mg/g fresh weight)	Chlorophyll 'b' content (mg/g fresh weight)	Total Chlorophyll content (mg/g fresh weight)	Carotenoids content(mg/g fresh weight)
1	control	5.67± 0.65 *	3.32± 0.24 ***	8.63± 0.20 *	2.35± 0.51 ***
2	25µM	6.38± 0.51 **	3.69± 0.28 *	9.32± 0.29 ***	1.66± 0.37 **
3	50µM	5.03± 0.41 *	3.39±0.23 ***	8.06± 0.12 *	1.35± 0.22 **
4	75µM	4.04± 0.66 ***	2.61± 0.20 *	6.36± 0.06 **	1.32± 0.13 ***
5	100µM	4.37± 0.39 **	1.35± 0.18 *	4.69± 0.11 **	1.04± 0.01 *
6	125µM	2.47± 0.62 *	1.05± 0.15 **	3.09± 0.23 **	0.946± 0.32 **

Values represent the mean SE. Means ± standard error. Means with the same letter within a column are not significantly different, according to Duncan's multiple range tests: *P<0.05; **P < 0.01; *** P< 0.001.

In plants, they cause numerous slighter or stronger toxic effects. They inhibit the vegetative growth and yield productivity (Sanita di Toppi and Gabbrielli 1999). Cobalt (Co) is a contaminant trace element in the soil polluted by the metal refineries near the agricultural area (Gad 2005)]. Nevertheless this trace element is necessary for the normal metabolic function of the plant. At high concentration, it is toxic and severely interfere physiological and biochemical functions. A complex antioxidant defence system is preventing the oxidative damages. It is developed by the plants to alleviate the damage caused by CoCl2. Plants containing this antioxidant system composed of low molecular weight antioxidants such as catalase (CAT) and peroxidases (POX). Proteins are compounds of fundamental importance for all function of the cell. Protein variation is an essential part of plant response to biotic and abiotic stress as well as for adaptation to environmental conditions (Vierstra, 1993). Plants to tolerate abiotic stresses are by biosynthesis of stress proteins is called as stress tolerant proteins. The stress proteins influences that accumulate in plant are response to salinity, metals, low temperature and other environmental pollutions (Close, 1996, 1997). All these factors adversely affect the plant development and productivity. The present investigation aims to evaluate specific effects of cobalt stress on growth, photosynthetic pigments, antioxidant responsible enzyme in cluster bean and protein level under CoCl2 stress treatment. Consequently, to determine the cluster bean plants have evolved exclusive mechanisms to compact with environmental stress.

RESULTS

Morphology and biomass content

At the higher concentration of $CoCl_2$, we observed that the metal stress caused a significant reduction in shoot length, root length, fresh weight and dry weight of cluster bean. In low concentration (25 µM) of $CoCl_2$, it induces the growth rate, as compared with control (Table 1).

Overall, metal stress impaired biomass accumulation. The different concentration of $CoCl_2$ treatment showed a significant difference existed in the biomass accumulation in fourteen day's old plants. At the end of the experiment, maximum level of stress treated leaf fresh weight and dry weight decreased as compared with their respective control (Table 1). $CoCl_2$ stress also led to chlorosis and necrosis on cluster bean plans. During our observations significant differences existed in the leaf morphology of both control and treated plants (Figure 1). On the other hand, under all high concentration (100 µM and 125µM) of treatments showed decline in leaf area compared to their corresponding control and minimum concentration (25 µM, 50 µM and 75 µM) of treated plants (Table 1). Maximum concentration of $CoCl_2$ stress induced inhibition of plant growth rate and moreover, from the fourteen days treated plants exhibited a gradual decrease of plant growth, compared to control (Figure 1).

Pigment analysis

Chlorophyll and carotenoids pigments decreased significantly in leaves. Total chlorophyll (Chl a+b) and carotenoid contents exhibit significant changes under low concentration (25 µM) of metal stress conditions, compared to controls (Table 2). The level of chlorophyll a and b was increased upto 14.5 - 16.3% at 25 µM concentration of $CoCl_2$ than control. The increased chlorophyll content at lower level of $CoCl_2$ was obviously due to the better growth. The level of Chlorophyll a and b was considerably reduced to 67.2% and 60 % respectively after 14 days. The decline phase was observed along with the increased concentration of $CoCl_2$ (Table 2). Therefore, the total chlorophyll (a+b) and carotenoids (x+c) contents of cluster bean plants was affected by $CoCl_2$ stress treatment at high concentration (75 µM, 100 µM and 125 µM). Additionally, the carotenoid content in cluster bean showed decreased gradually under low to high concentration of metal stress. However, the decreased in chlorophyll and carotenoids contents was only significant on the fourteen days old compared to

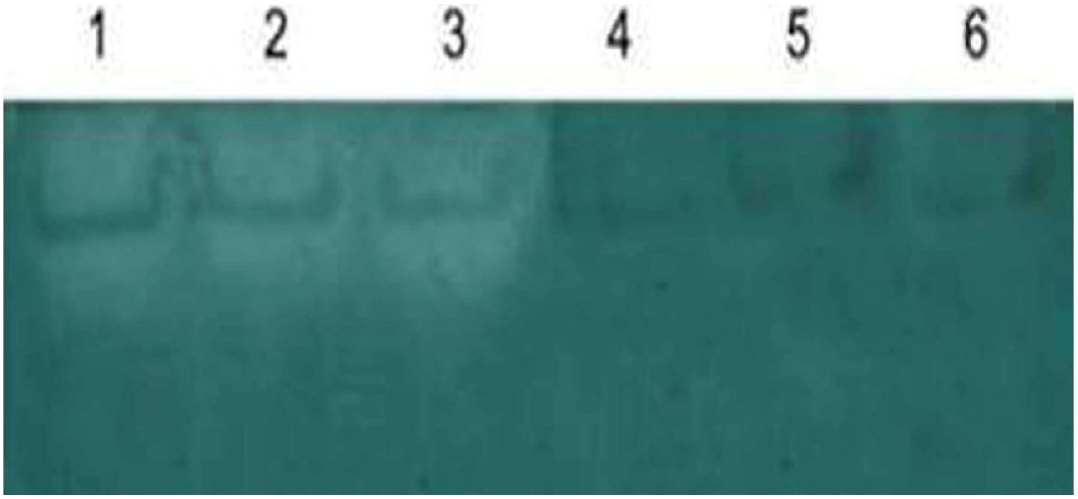

Figure 2: Activity staining and relative contribution of CAT in extract of leaves of *Cyamopsis tetragonoloba* L. exposed to different concentrations of $CoCl_2$ and ambient (25 - 125 μMol^{-1}) $CoCl_2$ treatments for 14 days. Samples applied to the gels contained 10 µg of protein. Lane 1 (control), Lane 2 (25 µM), Lane 3 (50 µM), Lane 4 (75 µM), Lane 5 (100 µM), Lane 6 (125 µM) respectively.

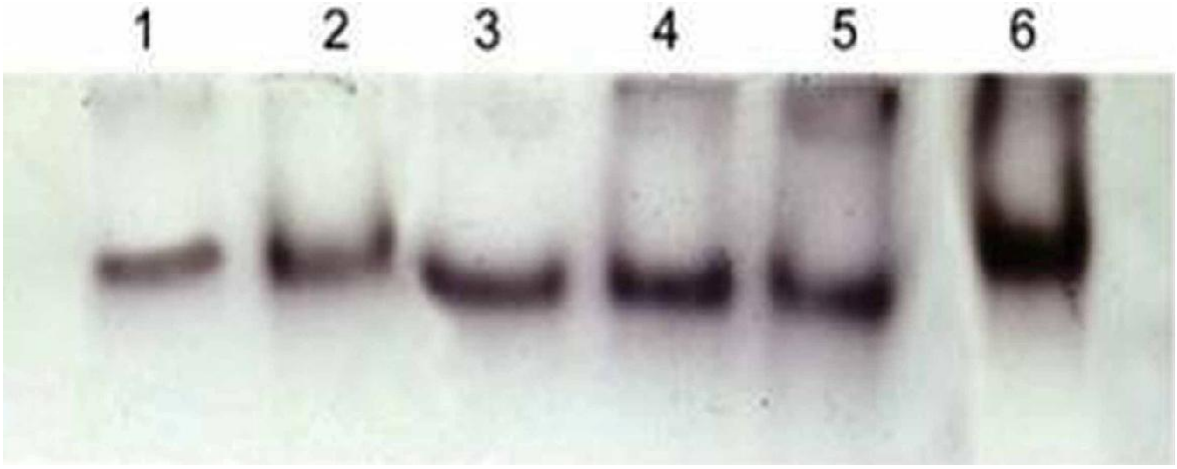

Figure 3. Activity staining and relative contribution of POX in extract of leaves of *Cyamopsis tetragonoloba* L. exposed to various metal concentrations and ambient (25 – 125 μMol^{-1}) $CoCl_2$ treatments for 14 days. Samples applied to the gels contained 10c µg of protein. Lane 1 (control), Lane 2 (25 µM), Lane 3 (50 µM), Lane 4 (75 µM), Lane 5 (100 µM), Lane 6 (125 µM) respectively.

corresponding control plants. The decline phase was observed along with increased concentration of $CoCl_2$ (Table 2).

Antioxidant enzymes extraction and assay

Significant decreases in CAT and POX activities were examined in fourteen days of old seedlings. Significant decrease was observed in leaves at higher concentration (75 µM, 100 µM and 125 µM) of $CoCl_2$ treatments. CAT activity showed significant change in control and low concentration (25 µm, 50 µM) of treated leaves as compared with their higher concentration (Figure 2). However in higher concentration did not exhibit significant changes and the two isozymes of CAT was not separated in gel electrophoresis (Figure 2). In fourteen days old leaves of control and minimum stress treated (25 µm and 50 µM) contain CAT activity at present in the form of two isozymes. The densitogram of two isozymes (CAT 1 and CAT 2) were analyzed under Lark gel imaging system (Figure 2). During separation of these isozymes on non-denaturing polyacrylamide gels, we observed that CAT1 and CAT 2 but CAT 2 was not very susceptible to oxidation on the gel. High concentration (75 µM, 100 µM and 125 µM) did not fully protect CAT1 and CAT 2

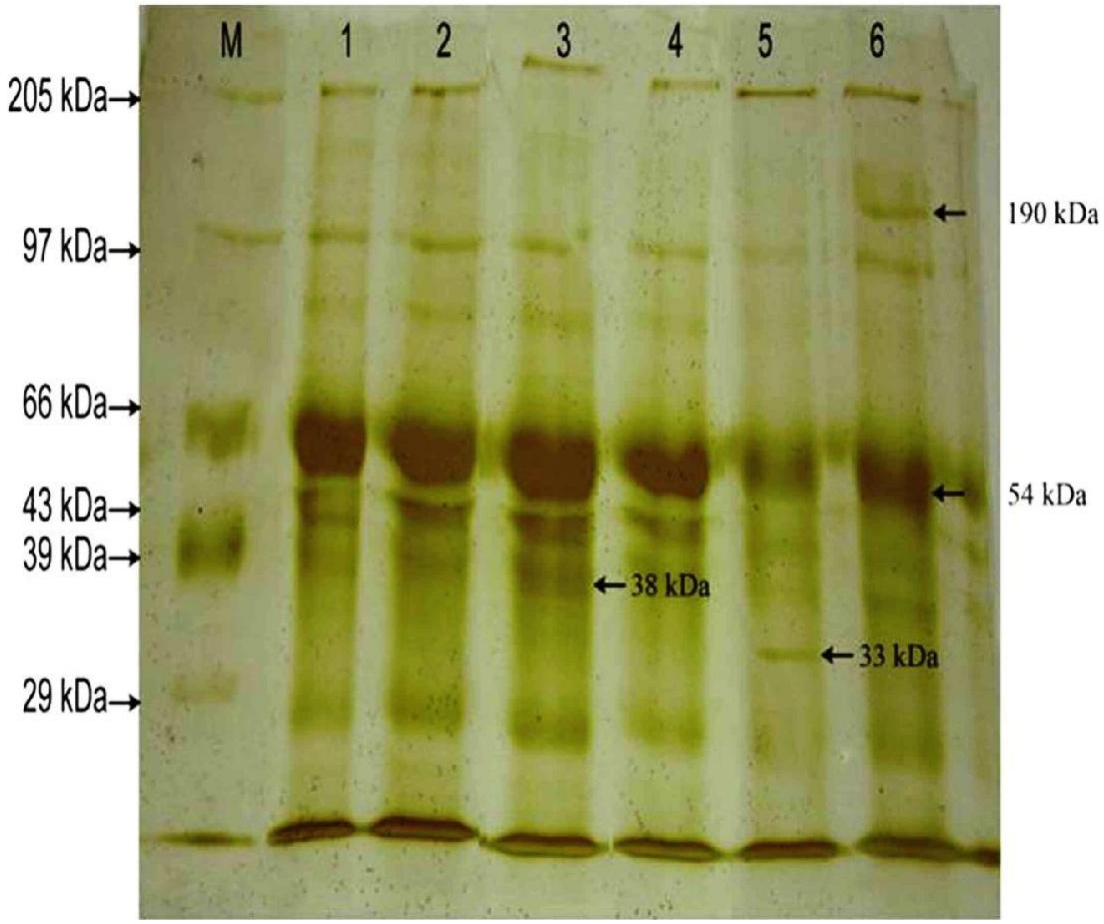

Figure 4. Polypeptide (SDS - PAGE) of total soluble proteins in the control and stress treated cluster bean plants. Lanes M – molecular markers (indicated from the left side of gel) Lane 1 – control (No treated), Lane 2 - to 6 represents (25, 50, 75, 100 and 125 µM) concentration of $CoCl_2$ treated samples. The crude protein extract 10 µg from *Cyamopsis tetragonoloba* L. (prepared as described in materials and methods) were loaded into each lane, respectively.

activity. DTT was found to protect CAT 1 but not significant effects on CAT 2 isozymes (Morikofer-Zwez et al., 1969)

We observed significant differences in total CAT activity (Figure 2) among the treated and control plant leaves. CAT activity was significantly higher in control and minimum stress treated leaves but not significant in maximum concentration. The contributions of two isozymes are closely reflecting the total activity of plants, whereas CAT 1 dominates in among control and minimum levels of $CoCl_2$ treated leaves. This result was consistent replicated in gels. Total POX activities (Figure 3) was generally lowest in control and minimum concentration treated (25 µM and 50 µM) leaves and significant inducing POX activity in maximum concentration (75µM, 100 µM and 125 µM) treated leaves. The densitogram of POX activity of control and treated plant samples were analyzed under Lark gel imaging system (Figure 3). According to the densitometry, high intensity of isozymes bands were observed on maximum concentration (75 µM, 100 µM and 125 µM) of CO stress treated plants. Whereas, the low intensities was obtained in control and minimum concentration (25 µM and 50 µM) of stress treated leaves. The intensity of POX isozyme was gradually increased along with the increased concentration of $CoCl_2$.

Protein analysis by SDS – PAGE

Total protein in leaves was analyzed by Bradford method and separated by SDS PAGE (Figure 4). The protein profile of cluster bean has shown high intensive protein bands under stress conditions. In stress treated ($CoCl_2$) plants exhibit numerous sets of intensive bands were

detected. Figure 4 shows the greatest number of polypeptides detected in control and stress treated plants. Four new bands (stress responsive protein) were synthesized in stress treated plants (190 KDa, 54 KDa, 38 KDa and 33 KDa). It is appeared on gel lane 3, 5 and 6. The most significant difference however, the induction of new bands respectively 190 KDa, 54 KDa, 38 KDa and 33 KDa polypeptide in stressed seedlings that was absent in the control plants (Fig 4).

DISCUSSION

The results of the present study confirm that metal stress causes a reduction in vegetative growth and decrease in total plant productivity. This may be due to inhibition of cell division, cell elongation or combination of both under metal stress. Biomass of leaves was higher at 25 µM concentration which gradually declined towards higher concentrations (50 µM to 125 µM). There are large number of reports that the low concentration of heavy metal stress increased the plant dry matter and yield productivity (Jayakumar and Vijayarengan, 2008). Thus, the results indicate that the higher concentration of metal plays an important role in plant productivity process. Biomass production depends on the accumulation of carbon products through photosynthesis (Panda et al., 2010), but elevated metal stress can adversely affect photosynthesis by altering chlorophyll content. Depletion in plant growth under abiotic stress is attributed to decreased water uptake followed by limited hydrolysis of food and impaired translocation of food reserves from the storage tissues to developing axis (Khedr et al., 2003; Demiral and Türkan 2005; Misra and Gupta, 2006).

Photosynthetic pigments such as chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents of *Cyamopsis tetragonoloba* decreased at high amount of Co level in soil. Similar changes in the photosynthetic pigment contents by various metal stresses were regarded (Rout and Das, 2003). The increased chlorophyll content at lower level of $CoCl_2$ was obviously due to the better growth. The mechanism of heavy metals on photosynthetic pigments may due to the three reasons: heavy metal enters into chloroplast and may be over accumulated in local causing oxidative stress, which will cause damages like peroxidation of chloroplast membranes (Clemens et al., 2002). In addition, they can directly destroy the structure and function of chloroplast by binding with -OH group of enzyme and overall chlorophyll biosynthesis through Mg_2, Fe_2 (Clemens et al., 2002). Heavy metals may activate pigment enzyme and accelerate decomposition of pigment. Impaired chlorophyll development by heavy metals may be due to the interference of protein; the treatments presumably blocked the synthesis and activities of enzymes responsible for chlorophyll biosynthesis. Thus, the results reveal that the excess supply of cobalt seems to prevent the incorporation of iron in photo porphyrin molecule resulting in the reduction of pigment composition.

Anti-oxidant, defense system was positively associated with the abiotic stress tolerance in plants. Plants have several physiological and biochemical strategies, such as antioxidative defense and osmotic adjustment, to prevent the damaging effect of oxidative stress resembling biotic and abiotic stress (Tan et al., 2006). The higher catalase activity in the stress tolerant lines would scavenge active oxygen radicals more effectively and thereby protect them against salt or drought stress - induced oxidative damages. We observed the induction of the two isozymes of CAT in cluster bean plants. Although the significance of POX as an H_2O_2 scavenger is clear but the role of CAT is not well recognized. These enzymatic studies reveal that the increase in metal concentration show an increase in peroxidase (POX) activities. This can be compared with earlier reports (Seliga, 1993). To identify the most reactive individual isozymes with substrate on stress treated leaves, this content with previously reported (Anderson et al., 1994). However the fact that those isozymes were elevated in cluster bean plants suggested a role in metal tolerance. The higher antioxidant enzyme activity has a role in imparting tolerance against any type of environmental stresses. This oxidative enzyme has also served as to catalyze the reduction of toxic intermediates of oxygen metabolism to prevent cellular damage (Jaleel et al., 2008).

We have observed that the effect of accumulation on antioxidant enzymes differs depending on concentration of metal stress in plant tissues. The cluster bean plant is visibly the most susceptible to CoCl2 stress damage, and we observe the induction of antioxidant enzyme. We also observe that the toltal CAT and POX activity (Fig 2 & 3) were induced in cluster bean and it is in strongly supporting the protectively against metal stress damages with induction of antioxidant enzymes during accumulation. These responses were confirmed by examination of isozymes profiles.

There was a higher total protein content in cluster bean leaves samples exposed to high concentration of CoCl2 stress treated. We also detected four new proteins were synthesized in stressed plants. Similar studies have been undertaken by earlier workers. The essential function of heat shock protein is preventing aggregation and assisting refolding of non active proteins under both stress and normal conditions (Hartl 1996; Frydman, 2001). (Zahran et al., 1994) reported the appearance of new protein bands in sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS - PAGE) profiles of rhizobia from woody legumes grown under salt stress. (Garcia et al., 2006) obtained protein bands with molecular weight 42 and 45 kDa on SDS-PAGE related to stress. (Parida et al., 2004) reported that the SDS –

PAGE analysis showed nearly identical protein profile in control and salt treated samples which suggest that salt did not alter protein synthesis. In our study we observed distinction protein synthesis patterns suggest that metal stress able to alter the protein synthesis or proteolytic activity. Additional new polypeptides might be considered as stress tolerant proteins. This new proteins may be considered as genetic construction and it is needed to for more understanding of its tolerant ability to metal stress.

This investigation helps to understand the response of plants to stress at physiological, biochemical and molecular levels. The morphological responses are the result of the molecular changes. We also observed to identify the antioxidant enzyme accumulation and intensive of total polypeptides in cluster bean. Few new polypeptides with molecular weight (190 KDa, 54 KDa, 38 KDa and 33 KDa) were found in $CoCl_2$ stress a treated sample that was high intensive and constitute a stress specific response like protector against metal stress.

MATERIAL AND METHODS

Plant materials and seed treatments

Cluster bean (*Cyamopsis tetragonoliba* L.) cultivar was used to evaluate the physiological and biochemical activities under stress at an early seedling stage. Seeds of cluster bean (*Cyamopsis tetragonoliba* L.) were surface sterilized in 0.1% $HgCl_2$ for 5 minutes and washed thrice thoroughly in distilled water. Seeds were then soaked in sterile water at 28°C for 12 hours and then planted on plastic pots, containing 1 kg of autoclaved sterile soil with maximum water holding capacity. Plants were grown in a green house condition with a temperature of 28 ± 2°C photoperiod 1500 µmol m^{-2} s^{-1} and relative humidity of 70 %. When the plants were 7 days old, the metal treatment was initiated (25, 50, 75, 100 and 125 µM 1^{-1}) in the form of $CoCl_2$. It was applied for 5 days frequently and the plant was determined under metal stress condition. The metal stress cause severe growth inhibition, photosystem and oxidative damages. The experiment was repeated with twice and data was recorded after 14 days.

Growth parameters

Growth parameters were determined at the end of stress treatment, shoot length was measured from the soil surface to the newly emerging leaf of the cluster bean. Root length and entire seedlings was measured (cm/plant). The fresh and dry weight of control and treated leaves were measured (g/plant) from randomly selected plants. It is used to evaluate the water status of the leaves. To each treatment, three leaflets from each replicate were used to analyse the relative water content. It was calculated according to (Farrant, 2000).

Pigment analysis

To determine the content of chlorophylls (a+b) and total carotenoids of leaves, 100 mg of leaf tissue from each of the three replicates were used. The pigments were extracted from leaf tissues in 1 ml of 100% ice cold acetone. The absorbance of extracts was measured at 662, 645 and 470 nm using a UV – Visible spectrophotometer. The content of chlorophylls (a+b) and caroteniods (x+c) were calculated using (Lichtenthaler, 1983) extinction coefficient method. The content of chlorophyll pigment is expressed as mg/g Fw^{-1}.

Antioxidant enzymes extraction and assay

The control and treated leaves were excised from the seedlings (0.1g) and homogenized with mortar and pestle at 4°C in 5 ml extracting buffer (0.1 M phosphate buffer, pH 6.8). The homogenate was then centrifuged at 15000 rpm for 15 min, the experiment carried out at 4°C and homogenized was used as the crude extract for the catalase (CAT) and peroxidase (POX), according to the method of (Beers Jr and Sizer, 1952). The CAT activity was determined spectrophotometrically by following the decline in A_{240} as H_2O_2. CAT isozymes were separated on non-denaturing ployacrylamide gels at 80 V for 2h at 4°C using procedure of (Laemmli, 1970). The gel was prepared by acrylamide with a quantity of N-N-methylene-bis-acrylamide ($CH_2(NaCHOCH{=}CH_2)_2$). The samples 20 µl (60µg) with 20 µl of tracking dye were loaded directly into the wells with a micropipette. Gels were then soaked in 3.27 mM H_2O_2 for 20 min, rinsed in water and stained in solution of 2% (w/v) potassium ferricyanide, 2% (w/v) ferric chloride (equal volume of each component added sequentially), according to the method of (Anderson et al., 1995).

Total POX activity was determined spectrophotometrically by following the presence of H_2O_2 according to the method of (Britton and Mehley, 1955). POX isozymes were separated on non-denaturing acrylamide gels. The gel was run at 20 V constant powers for 4 h at 10°C, after electrophoresis the. gel was stained with p-Phenylenediamine (0.1 M sodium citrate, pH 5.0, 9.25 mM p-Phenylenediamine) and 3.92 mM H_2O_2 for 10-15 min, according to the method of (Olson and Varner, 1993). The POX activity was observed at the cathode strip, bands were confirmed by examination of isozyme profiles.

Protein extraction and analysis by nondenaturing ploy acrylamide gel electrophoresis (native PAGE)

Fresh tissue (0.1g) was homogenized in 1.0 ml of chilled 50 mM potassium phosphate buffer (pH 7.0) in chilled mortar and pestle. The homogenate was incubated for 4h at 4°C and then centrifuged at 15000 rpm for 15 min at 4°C. The supernatant was collected and the protein concentration of the crude extraction was determined by spectrophotometrically. Bovine serum albumin (BSA) was used to prepare a standard curve, according to the methods of (Bradford, 1976).

The samples (30 µg) of protein were subjected to PAGE (polyacrylamide gel electrophorosis) as described in (Laemmli 1970). The gels used for native PAGE were 4 - 20% gradient acrylamide cast with a gradient mixer. Extracts were subjected to electrophoresis at in 5 % stacking and 10 % separating gels. The gels were run at a constant of 100V at 4°C with a vertical electrophoresis apparatus (BIORAD). The protein bands were washed twice for 2 min with deionized water and fixed for 30 min in a solution containing 50 % (v/v) ethanol, 12 % (v/v) acetic acid and 0.055 % (v/v) formalin. After fixation, the gels were washed two or three times in deionized water and stained in 0.2% (w/v) silver nitrate for 30 min. After staining, the gels were washed three times in deionized water and then developed in solution containing 6 % sodium carbonate and 0.05% formalin until protein band were developed. Gels were then soaked in 12 % v/v acetic acid to stop the staining process. The molecular weight of the separated polypeptides was determined and the elctrophorotic protein bands are exhibit in cluster bean samples. It was subjected to protein analysis according their molecular weight as describe by (Eissa, 2009).

Statistical analysis

All the experiments were repeated twice with three replicates (n=3) and data presented are mean ± S.E. The results were subjected to ANOVA using GLM factorial model on all the parameters.

Duncan's test was used for comparison between the treatments. The data analysis was carried out using statistical package SPSS 7.5 com-parison with $P < 0.05$ was considered significantly different.

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