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RESEARCH ARTICLE

Copper-stress induced alterations in protein profile and antioxidant enzymes activities in the *in vitro* grown *Withania somnifera* L.

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Abstract *Withania somnifera* L. seedlings were grown in half-strength MS (Murashige and Skoog) basal medium for 4 weeks and then transferred to full-strength MS liquid medium for 3 weeks. The sustainable plants were subcultured in the same medium but with different concentrations (0, 25, 50, 100 and 200 μM) of Cu for 7 and 14 days. The growth parameters (root length, shoot length, leaf length and total number of leaves per plant) showed a declining trend in the treated plants in a concentration dependant manner. Roots and leaves were analyzed for protein profiling and antioxidant enzymes [catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1) and guaiacol peroxidase (GPX, EC 1.11.1.7)]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of crude protein extracts showed the appearance of some new proteins due to Cu treatment. In plant samples grown with 25 and 50 μM of Cu, a rapid increase in antioxidant activities were noticed but at higher concentration (100 and 200 μM) the activities declined. Isoforms of CAT, SOD and GPX were separated using non-denaturing polyacrylamide gel electrophoresis and concentration specific new isoforms were noticed during the study. Isoforms of the antioxidant enzymes synthesized due to Cu stress may be used as biomarkers for other species grown under metal stress.

Keyword Antioxidant enzymes · Copper stress · Protein profiling · SDS-PAGE · *Withania somnifera* L.

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Introduction

Copper (Cu) is an essential redox active transition element in biological systems and is involved in many physiological processes in plants. Copper plays an important role in signaling of transcription and protein trafficking machinery, oxidative phosphorylation, hormone signaling and iron mobilization (Yruela et al. 2000; Raven et al. 1999). It acts as the cofactor in many enzymes such as Cu/ Zn SOD, cytochrome c oxidase, amino oxidase, laccase, plastocyanin and polyphenol oxidase (Halliwell and Gutteridge 1984). However, Cu induces toxicity at tissue concentrations slightly above its optimal levels (Yruela 2005; Halliwell and Gutteridge 1999). Studies from some plant species demonstrate that excess Cu in the plant growth medium induced alterations in the photosynthetic and respiratory processes, enzyme activity, DNA and membrane integrity, all of which could lead to growth inhibition (Salt et al. 1998). Excess of heavy metals may stimulate the production of free radicals and reactive oxygen species (ROS) such as

superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radicals ($OH^{\bullet}$) (Sharma et al. 2012; Yruela 2005; Ercal et al. 2001). The ROS has a role in lipid peroxidation, membrane damage and consequently in plant senescence (Halliwell and Gutteridge 1999; Fridovich 1986). Antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) are involved in the scavenging of ROS (Gill and Tuteja 2010). SOD is a metalloprotein that catalyzes the dismutation of superoxide to H_2O_2 and molecular oxygen. CAT is found predominantly in peroxisomes, which dismutates H_2O_2 into H_2O and O_2, whereas POX decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and or antioxidants (Halliwell and Gutteridge 1999; Fridovich 1986).

Withania somnifera (L.) Dunal is an important medicinal plant that belongs to the family Solanaceae. It is popularly known as Ashwagandha or Winter Cherry (Weiner and Weiner 1994). It is described as a herbal tonic and health food in Vedas and considered as 'Indian Ginseng' in traditional Indian system of medicine (Singh et al. 2001). The different parts of the plant are generally used as anti-inflammatory, anticancer, anti-stress and immune-modulator, adaptogenic, central nervous system, endocrine and cardiovascular activities (Alam et al. 2011). The root extracts significantly reduce the lipid peroxidation and increase the SOD and CAT activity, thus possess a free-radical scavenging property (Dhuley 1998). The metal-accumulating plants are now being employed to remove the excessive metals from soil and aqueous streams (Salt et al. 1998). The metal uptake process and accumulation by different plants depend on the concentration and solubility of available metals in soil and the plant species growing on those soils. However, in addition to the knowledge of uptake, translocation, or compartmentation of heavy metals in plants, understanding the tolerance mechanisms to improve plants of biotechnological interest is also important (Salt et al. 1998; Zenk 1996). The present investigation was therefore undertaken to determine the specific proteins induced by Cu stress with proteomic approaches and to study the effect of Cu on antioxidant enzymes (CAT, SOD and GPX) of the *in vitro* grown plants of *W. somnifera*.

Materials and methods

Plant material and culture condition

Seeds of *Withania somnifera* L. were collected from botanical garden, Post Graduate Department of Botany, Utkal University, Bhubaneswar, Odisha, India, and washed thoroughly under running tap water for 30 min followed by the treatment with an aqueous solution of 5 % Teepol (Reckitt's Colman, Kolkata, India) for 10 min and rinsed five times with double distilled water. The seeds were then surface disinfected with an aqueous solution of 0.1 % $HgCl_2$ (Hi-Media, Mumbai, India) for 5 min and rinsed five times with autoclaved double distilled water. The disinfected seeds were inoculated in 150 ml Erlenmeyer flasks (Borosil, Bangalore, India) containing half-strength MS basal medium (Murashige and Skoog 1962), with 0.7 % (w/ v) agar (Hi-media, Mumbai, India). The pH of the medium was adjusted to 5.8 ± 0.2 before autoclaving at 121 °C for 15 min. The seeds were germinated at 25 ± 1 °C, 60 % relative humidity and 2,000 photon flux intensity provided by cool white fluorescent tubes (Philips, Bangalore, India). The germinated seedlings were grown for 1 month in the same medium and subsequently transferred to MS liquid medium (control) or MS liquid medium containing different concentrations (0, 25, 50, 100 and 200 μM) of Cu in the form of $CuSO_4$ for 7 and 14 days.

Growth parameters

After 7 and 14 days of treatment, plants were carefully removed from the MS liquid medium and washed twice with distilled water. The plants were blotted dry with paper towels and then the various morphological parameters such as root length, shoot length, leaf length and total number of leaves per plant were taken and recorded.

Extraction and estimation of total soluble protein

For estimation of total soluble protein, leaf and root tissues were extracted by the modified acetone-trichloroacetic acid (TCA) precipitation method of Damerval et al. (1986). Leaf and root tissues were homogenized separately with cold 50 mM sodium phosphate buffer (pH7.8) using pre-chilled mortar and pestle. The resulting homogenates were centrifuged at 4 °C at 14,000 g for 15 min (Remi Instruments, India). The supernatants were mixed thoroughly with equal volume of 10 % TCA, kept overnight at 4 °C to facilitate complete precipitation of soluble protein. The precipitates were then centrifuged at 12,000 g for 10 min and the pellets were washed with 100 % acetone in order to remove the pigments. The pigment-free pellets were washed successively with 80 % ethanol, 3: 1 (v: v) ethanol: chloroform, 3: 1 (v: v) ethanol: diethyl ether and finally with diethyl ether to remove phenolic compounds. The washed pellets were air dried and solubilized by resuspending in a known volume of 0.1 N NaOH for 16 h at 37 °C. The samples were centrifuged and supernatants collected for protein estimation. Quantitative estimation of protein was done according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Analysis of protein profile by SDS-PAGE

Leaf and root samples of control and treated plants were homogenized with 50 mM potassium phosphate buffer (pH

7.8), 10 % (w/ v) insoluble polyvinylpyrrolidone (PVP) and 2 mM phenylmethylsulfonyl fluoride (PMSF) in pre-chilled mortar and pestle. The homogenate was centrifuged at 14,000 g for 15 min at 4 °C and the protein concentration was estimated (Lowry et al. 1951). The equal amounts (50 μg) of proteins were separated on 5 % stacking and 10 % resolving polyacrylamide slab gels (Laemmli 1970) at a constant current of 35 mA for 4 h. Separated polypeptides on the gel were visualized by the silver staining method (Switzer 1979). The gels were then scanned and photographed by gel documentation system and analyzed with quantity one software from Bio-Rad (Bio-Rad, Italy). In order to achieve the precise sizing of the separated polypeptides, a protein molecular weight marker [phosphorylase b (97.4 kDa); bovine serum albumin (66.0 kDa); ovalbumin (43.0 kDa); carbonic anhydrase (29.0 kDa); lactoglobulin (18.4 kDa)] was used as standard.

Preparation of enzyme extract

Frozen tissues of root and leaf (3:1 buffer volume:fresh weight) were homogenized in pre-chilled mortar and pestle with 50 mM potassium phosphate buffer (pH7.8), 50 mM ethylenediamine-tetraacetic acid (EDTA), 2 mM PMSF and 10 % (w/ v) insoluble PVP to fine slurry followed by centrifugation at 14,000 g for 15 min at 4 °C. The supernatants were desalted by passing through gel filtration columns, packed with pre-swollen Sephadex G-25 (Sigma, USA). The eluted fractions were tested for antioxidant assay. The fractions responding to the assay were pooled and stored at −20 °C for assay of CAT, SOD and GPX.

Assay and activity staining of CAT

CAT activity was determined by measuring the decrease in the H_2O_2 concentration at 240 nm (Aebi 1983) and the activity was calculated by using the extinction coefficient of 40.0 mM/ cm for H_2O_2 at 240 nm. Activity was assayed spectrophotometrically by taking 50 μg of protein at 25 °C in a reaction mixture containing 2.0 ml of 100 mM sodium phosphate buffer (pH6.8), 0.5 ml of 10 mM H_2O_2. The specific activity was expressed as nkatal per mg of protein.

Non-denaturing PAGE was carried out with 10 % resolving and 5 % stacking gel at 120 V for 12 h at 4 °C. Enzyme samples corresponding to 100 μg protein mixed with glycerol were layered on top of the stacking gel. CAT isoenzymes were determined as described by Woodbury et al. (1971). The gels were incubated in 0.003 % H_2O_2 for 10 min and developed in a reaction mixture containing 2 % (w/ v) ferric chloride and 2 % (w/ v) potassium ferricyanide for 10 min. The isoenzymes appeared as colourless bands on a deep blue background on the gel.

Assay and activity staining of SOD

The activity of SOD was assayed by measuring the inhibition of superoxide-driven nitrite formation from hydroxylamine hydrochloride according to Das et al. (2000). The reaction mixture was prepared by mixing 1.110 ml of 50 mM phosphate buffer (pH7.8), 0.075 ml of 20 mM L-methionine, 0.040 ml of 1 % (v/ v) Triton X-100, 0.075 ml of 10 mM hydroxylamine hydrochloride and 0.1 ml of 50 μM EDTA. To this mixture 100 μl of enzyme extract (50 μg protein) and 80 μl of riboflavin (50 μM) were added. The reaction was started by exposing the mixture to cool white fluorescent light for 10 min. After this period the light was swithed off, 1 ml of Greiss reagent (prepared freshly by mixing equal volume of 1 % sulphanilamide in 5 % phosphoric acid and 0.1 %N-1-napthyl ethylene diamine) was added to each tube and the absorbance was measured at 543 nm. One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50 % of nitrite formation. The enzyme activity was calculated from the value of V_0/ V-1, where V_0 is the absorbance of the control (without enzyme) and V is the absorbance of the sample. The activity was expressed as units per mg of protein.

SOD was localized on 10 % native gel using the photochemical method of Beauchamp and Fridovich (1971). The gels were incubated in staining buffer containing 50 mM potassium phosphate buffer (pH7.8), 0.1 mM EDTA, 28 mM TEMED, 0.003 mM riboflavin and 0.25 mM nitro blue tetrazolium (NBT) for 30 min in the dark at room temperature. The gels were then placed on a clean glass plate and illuminated by two fluorescent tubes (20 W each) until the SOD activity bands became visible. The bands appeared as light bands on a violet background.

Assay and activity staining of GPX

GPX activity was assayed according to the method and techniques followed by Bergmeyer (1974). Reaction mixture in a total volume (3 ml) consisting of 2.8 ml of 100 mM potassium phosphate buffer (pH7.0), 50 μl of 0.018 M guaiacol and 50 μl of 10 mM H_2O_2. The reaction was started by the addition of enzyme extract (100 μl) equivalent to 50 μg protein. The change in absorbance at 436 nm due to the oxidation of guaiacol to form tetraguaiacol in the presence of H_2O_2 was measured. The activity was calculated as enzyme units per mg of protein by using extinction coefficient of 26.6 mM/ cm due to tetraguaiacol formation.

Activity staining of GPX was performed on 10 % native PAGE according to staining procedure of Hamill and Brewbaker (1969). The gels were immersed for 30 min at room temperature in 0.018 M guaiacol, rinsed twice with deionized water, and then incubated in a solution of 0.015 % H_2O_2 in 1 % acetic acid. Reddish brown bands were

developed in about 10 min. Then it was rinsed with distilled water and scanned immediately (within 10 min).

Native gels stained for activities of CAT, SOD and GPX were scanned on a Bio-Rad gel imaging densitometer model GS-690 using the software "Quantity one" from Bio-Rad.

Statistical analysis

All results are the mean of three independent experimental replicates (n=6). The data were analyzed by analysis of variance (ANOVA) and tested for least significance differences (LSD) by Duncan's multiple range test at $P \leq 0.05$.

Results

Growth parameters

The freshly collected seeds of *W. somnifera* germinated satisfactorily in the basal half-strength MS medium within two weeks of inoculation. Healthy and uniformly grown plants were taken for further experiment. The adverse effects on various morphological parameters were detected in low concentration (25 μM) of Cu. After 7 and 14 days of aqueous exposure of Cu, there was considerable reduction in root length, shoot length, leaf length and number of leaves per plant. An overall inhibition was observed in higher concentration of Cu but the maximum diminution was found in 200 μM in both 7 and 14 days of treatment (Table 1).

Protein content and SDS-PAGE analysis

The total soluble protein content was found to be 2-fold higher in leaf samples of 7 days treated plants than 14 days. In roots, the maximum content of protein was found in 14 days treated plants (Fig. 1). When analyzed in SDS-PAGE, the total number of protein bands were found to be the same (9 and 7 respectively) both in leaf samples of 7 and 14 days treated plants and similar results were also observed in case of root samples. After the 7th day of treatment, there was a slight increase of polypeptides (10 and 9) in the leaf samples of 25

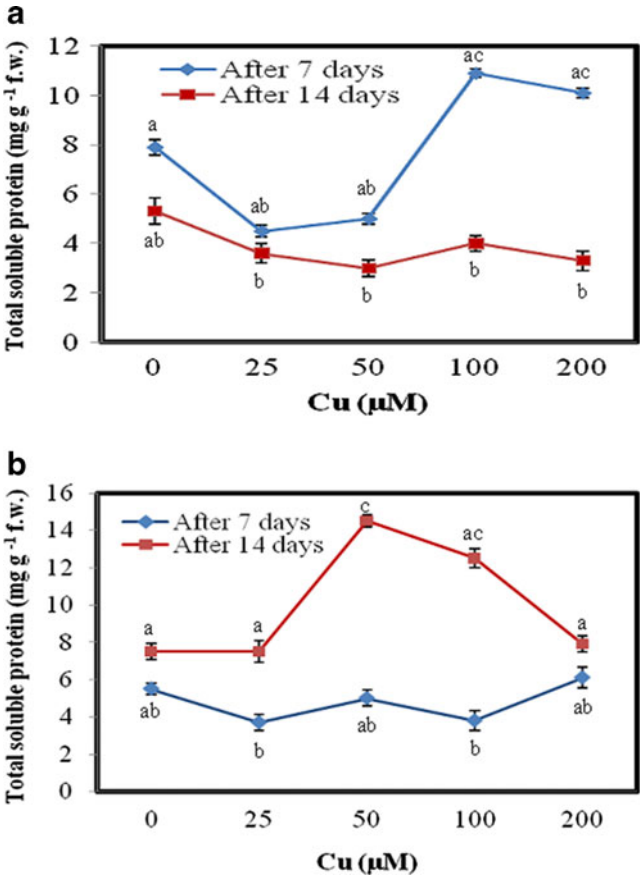

Fig. 1 Total soluble protein estimation under different Cu treatment (0, 25, 50, 100 and 200 μM) in leaf (**a**) and root (**b**) samples of *W. somnifera* L. The data plotted represent mean±SE of replicates (n=6). Symbol carrying different letters are significantly different at $P \leq 0.05$ by Duncan's multiple range test

Table 1 Growth parameters of treated and non-treated plants of *W. somnifera* L. under Cu stress

Treatment (μM)	Root length (cm)	Shoot length (cm)	Leaf length (cm)	No. of leaves/ plant
Growth parameters after 7 days				
0	6.74±0.22[a]	16.46±0.33[a]	1.30±0.21[a]	11.00±0.51[a]
25	6.50±0.34[a]	15.55±0.21[a]	1.22±0.16[a]	10.00±0.45[a]
50	6.23±0.19[a]	13.60±0.43[b]	0.97±0.22[b]	10.00±0.23[a]
100	5.13±0.22[b]	12.98±0.14[b]	0.91±0.37[b]	8.00±0.18[b]
200	4.56±0.26[b]	11.99±0.30[b]	0.78±0.12[b]	7.00±0.11[b]
Growth parameters after 14 days				
0	7.25±0.27[a]	18.45±0.66[a]	1.44±0.13[a]	14.00±0.62[a]
25	7.07±0.17[a]	14.49±0.34[b]	1.11±0.32[a]	12.00±0.54[a]
50	6.33±0.23[a]	11.34±0.44[b]	1.04±0.20[a]	11.00±0.51[a]
100	5.82±0.22[b]	8.35±0.52[c]	0.98±0.15[b]	9.00±0.46[b]
200	5.71±0.29[b]	8.33±0.25[c]	0.86±0.10[b]	9.00±0.22[b]

The data represent mean±SE of replicates (n=6). Values in the same column carrying different letters are significantly different between treatments and control at $P \leq 0.05$

and 50 μM Cu treated plants (data not shown). It was observed that a range of very high to low molecular weight polypeptides (148, 131, 113, 98, 87, 39, 29 and 19 kDa) were newly synthesized in both 25 and 50 μM Cu treated plants for 7 days treatment. But the expression of some polypeptides (101 and 95 kDa) reduced severely in Cu treated samples compared to control. Similarly, the leaves synthesized some new polypeptides like 52, 45, 39, 26, 20 and 15 kDa in 14 days treated plants. Protein band of 76 kDa was completely disappeared in all treated leaf samples of day 14 plants (data not shown). Root samples comparatively yield more polypeptides than leaves. In response to Cu, the 7 days treated (25 μM) plant roots yielded 145, 124, 77, 66, 27 and 18 kDa polypeptides. In higher concentration of Cu, no such bands were observed in the gel. Polypeptides like 90, 78, 71 and 18 kDa were visualized in 50 μM Cu of 14 days treated plants. A high molecular weight polypeptide (145 kDa) was obtained in 25 and 50 μM concentration of Cu in 7 days treated root samples (data not shown).

CAT activity

The activity of CAT was higher in the roots than the leaves of control plants during the experiment. With increasing Cu concentration (up to 50 μM) , a sharp increment of CAT activity was observed in both tissue samples of 7 days treated plants which was 2-folds more in comparison to control (Fig. 2a). But in 14 days old plants, the maximum activity was observed at 25 μM of Cu in leaf and root tissues, which was 2.66 and 2.81-folds increased respectively, over the control and then declined (Fig. 2b). These results were confirmed by assaying CAT activity in non-denaturing PAGE. A single isoenzyme of CAT was observed in both the samples of control and all the concentrations (25–200 μM) of Cu treated plants, but the intensities of band were varied from one sample to another (Fig. 2c, d).

SOD activity

Similar results were obtained in case of SOD activity which was higher in roots than the leaves of treated plants but the major difference is that the SOD activity was significantly increased in leaf and root samples at 50 μM of Cu both in 7 and 14 days of treatments. The leaf extracts of 7 days treated plants showed no major changes in SOD activities, but a significant increase was found in 50 μM of Cu after 14 days of treatment, which was almost 5-times higher than control.

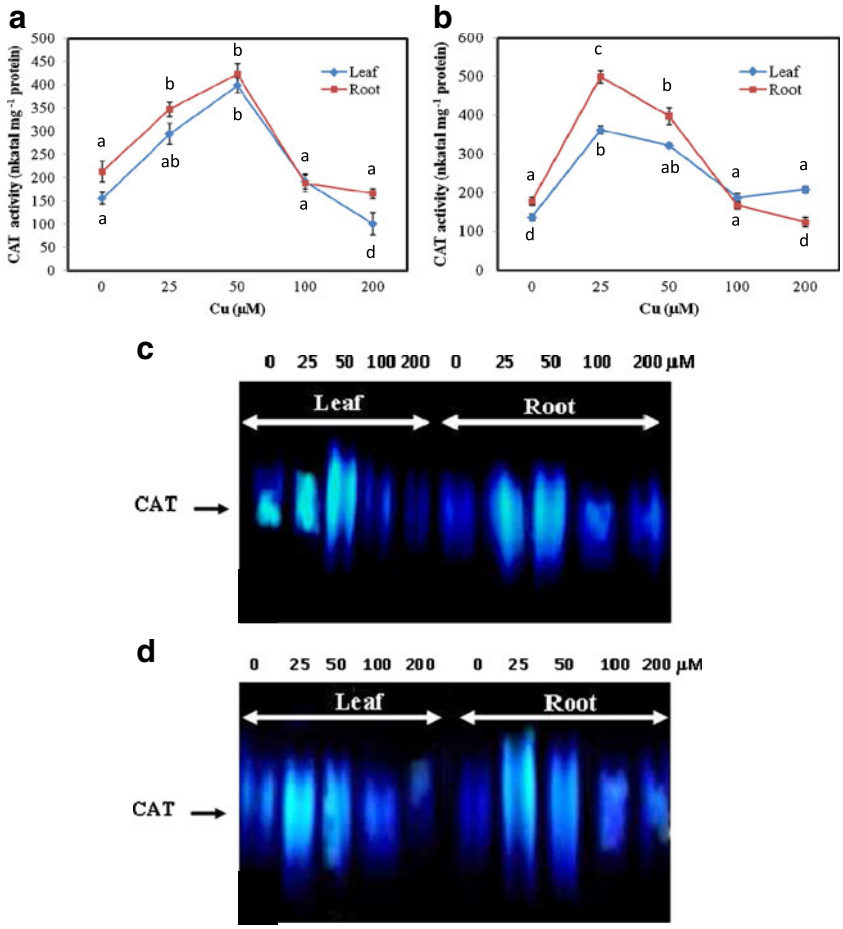

Fig. 2 CAT activity (**a**, **b**) and CAT isoenzymes (**c**, **d**) detected in extracts of *W. somnifera* L. grown for a 7 days (**a**, **c**) and 14 days (**b**, **d**) period on liquid MS medium containing different concentrations of Cu (0, 25, 50, 100 and 200 μM). Statistical analysis was as in Fig. 1

In case of root tissues, SOD activity increased 3-fold in 14 days old plants treated with 50 μM Cu (Fig. 3a, b). SOD activity decreased above the 50 μM Cu concentration. Several clear bands of SOD were observed in both the leaf and root tissues (Fig. 3c, d). In 7 days of treatment, two clearly visible bands (SOD-1 and SOD-2) observed in leaf samples of control plants but a new isoform (SOD-3) appeared on all treated plants (Fig. 3c). In root samples of 7 days treated plants, SOD-1 disappeared at a concentration of 200 μM. When the plants were exposed to 50 μM Cu for 14 days, leaves showed three bands (SOD-1, SOD-2 and SOD-3) whereas, the control sample gave a single band (SOD-2) and rests yield two bands. The two major isoforms (SOD-2 and SOD-3) were observed in roots of 14 days treatment but the SOD-2 band exhibited a slight widening in 25 and 50 μM of treatment (Fig. 3d).

GPX activity

Figure 4 shows the changes in the activity of GPX during the exposure of plants to varying concentrations of Cu. After 7 and 14 days of treatment, the leaf samples yielded more GPX activity than the roots. Under 50 μM Cu stress condition both 7 and 14 days treated leaves showed 5 and 24 times more activity respectively than the control. The root tissues showed maximum activity in 100 μM Cu after 7 days of treatment. Both 7 and 14 days treated (50 μM) leaf tissues gave maximum activity (Fig. 4a, b). The electrophoretic profiles of GPX isoenzymes showed three isoformic bands (GPX-1, GPX-2 and GPX-3) in both the leaf and root samples of 7 days treated plants except 200 μM of root tissues. When the plants were treated for 14 days the leaf and root tissues supplemented with 50 μM Cu showed three and two isoenzymes respectively (Fig. 4c, d).

Discussion

There was a reduction in all the morphological growth parameters at various concentrations of Cu and this may be due to the toxic effect of Cu at higher concentrations (Table 1). Though Cu is an essential micronutrient for normal plant growth and metabolism, but it can be toxic to the plants at higher concentration (Yruela 2005). It has been reported that, excess of Cu can inhibit the growth of plant height, root elongation and causes damage to root epidermal cells (Tanyolac et al. 2007; De Vos et al. 1989). Based on

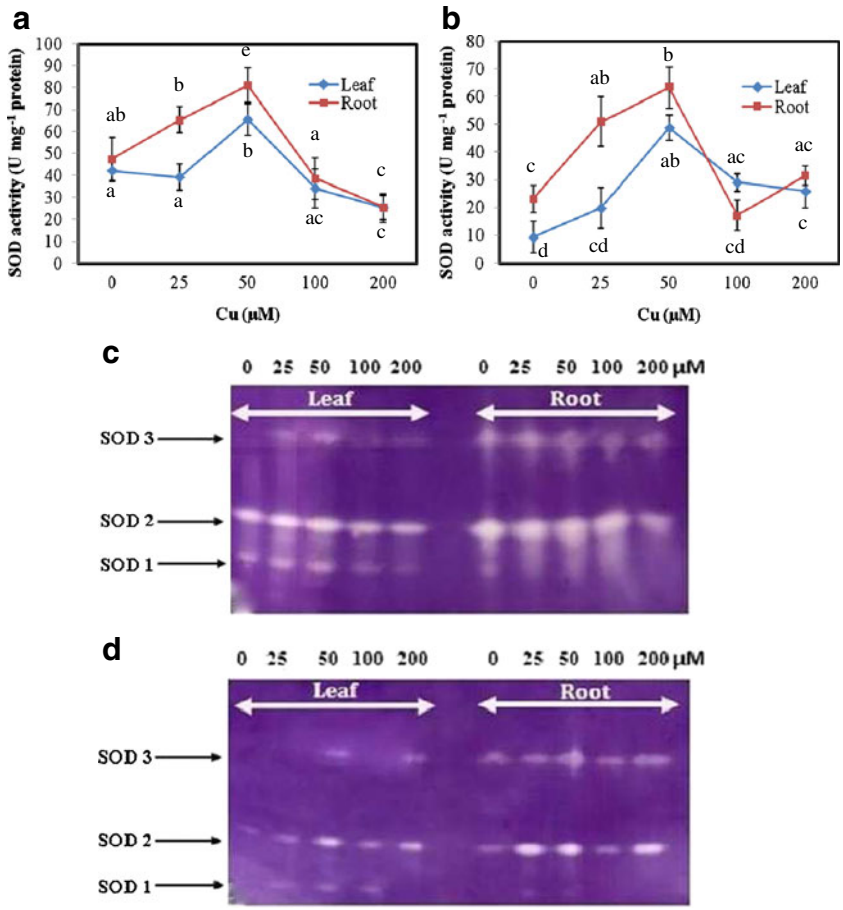

Fig. 3 SOD activity (**a**, **b**) and SOD isoenzymes (**c**, **d**) detected in extracts of *W. somnifera* L. grown for a 7 days (**a**, **c**) and 14 days (**b**, **d**) period on liquid MS medium containing different concentrations of Cu (0, 25, 50, 100 and 200 μM). Statistical analysis was as in Fig. 1

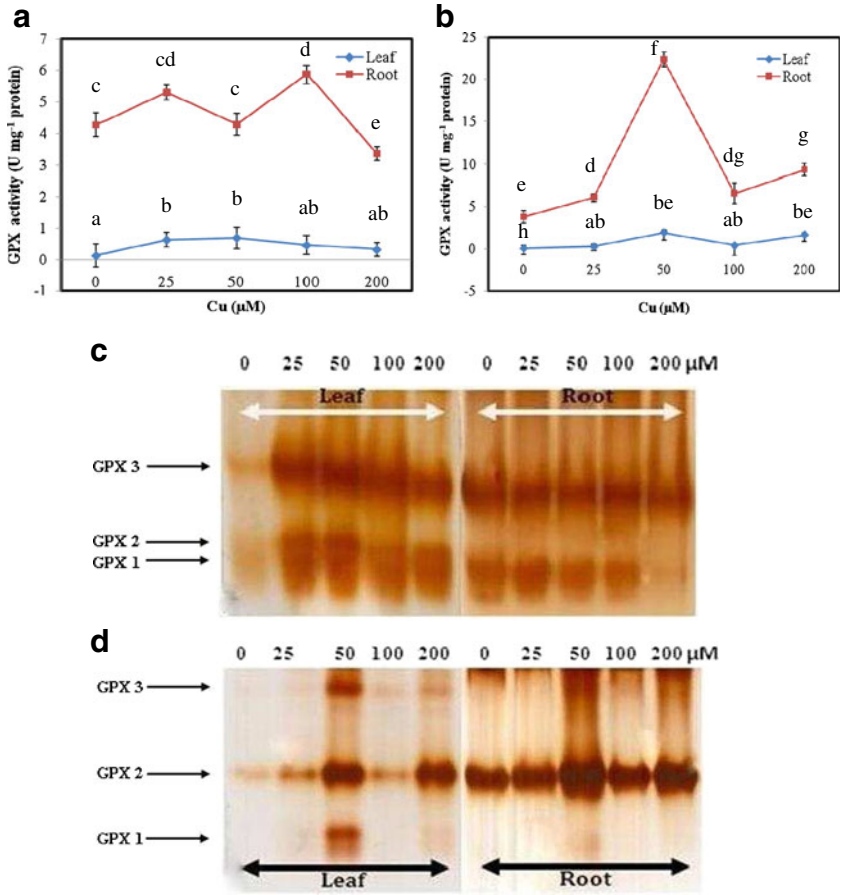

Fig. 4 GPX activity (**a**, **b**) and GPX isoenzymes (**c**, **d**) activity detected in extracts of *W. somnifera* grown for a 7 days (**a**, **c**) and 14 days (**b**, **d**) period on liquid MS medium containing different concentrations of Cu (0, 25, 50, 100 and 200 µM). Statistical analysis was as in Fig. 1

these results, our findings suggest that Cu concentration beyond 50 µM can inhibit the normal growth and development of *W. somnifera* under *in vitro* condition.

Protein content in treated leaves and roots increased significantly as compared to the control, and the highest increment (14.2±0.2 mg) was observed with 50 µM of Cu in the root samples of 14 days treated plants (Fig. 1). Soluble protein content in plants is known to respond to a wide variety of stress such as natural and xenobiotic (Sing and Tewari 2003). In this study, the content of soluble protein was increased by Cu stress up to a certain limit and then declined. The present investigation was also supported with the findings of Gao et al. (2008) that the high levels of Cu enhanced the protein content in leaves, roots and shoots of *Jatropha curcas*. The mechanism by which Cu affects protein content is complex and needs a further study.

The disappearance of some proteins and the de novo synthesis of others, in response to Cu-stress, indicated that such treatments are highly effective in causing a major re-shuffle of protein profiles of *W. somnifera* plant. However, several polypeptides were newly expressed in both leaves and roots of Cu treated plants (data not shown). Similar observations have been reported by Li et al. (2009) in *Elsholtzia splendens* under supplementation of increasing amount of Cu by performing both one and two dimensional gel electrophoresis. Since proteins were newly synthesized under Cu-stress, it appears to have a role in the mechanism of Cu tolerance which allows making biochemical and structural adjustments that enable the plant to cope with stress conditions. A remarkable observation was also noticed in 14 days treated leaf samples that the peptide like 76 kDa was totally absent in all the treated plants which coupled with the appearance of chlorosis, and root and leaf disintegration (data not shown). The results were supported with El-Khatib et al. (2011) who studied on aquatic macrophytes by taking increased concentrations of cadmium. However, early literatures suggest that the metal-binding ligands like metallothioneins (MTs) and phytochelatins (PCs) regulate the synthesis of metal-binding proteins/ polypeptides in plant under metal stress, which develop a clear understanding to optimize the process of metal tolerance in plant (Cobbett 2000; Tomsett and Thurman 1988).

It is well-known that Cu^{2+} is required by biological systems as a catalytic enzyme component but excess amount of Cu causes physiological responses that can decrease plant growth and develop toxic symptoms (Salt et al. 1998; Ouzounidou et al. 1992). Higher concentration of Cu can induce the formation of harmful ROS, leading to lipid peroxidation. This indicates that the oxidative stress is created in plant cells due to the overloading of metals (Gupta et al. 1999; Weckx and Clijsters 1996). SOD, CAT and POX

are important antioxidative enzymes that function in the cells to prevent the build-up of ROS. SOD dismutates superoxide, forming H_2O_2 which in turn may be detoxified by CAT and POX. As a result, the formation of the hydroxyl radical is prevented since it is produced by the interaction of superoxide and H_2O_2 being catalyzed by transition metal ions (Halliwell and Gutteridge 1999; Weckx and Clijsters 1996). Opposite results are reported where the activities of these enzymes decreased with the increase in heavy metal concentration; this can be caused by either the direct action of ROS on the proteins or on the inhibition of protein synthesis (Yruela 2005). Our results show both the effects for CAT activity. The activities measured after 7 and 14 days of treatment, there was an increase in activity up to a certain Cu concentration (50 μM), probably to eliminate the excess H_2O_2 induced by Cu (Saha et al. 2012: Dat et al. 2000), but at higher concentration or longer exposure time, the activities return to control levels or even lower (Figs. 2, 3, 4a, b). This can be caused by the negative effect of Cu-induced ROS on the enzyme proteins. Similar observation was observed in bean leaves (Weckx and Clijsters 1996). The activation/ inhibition processes of antioxidant enzymes are directly dependent on duration of the heavy metal exposure to plants. SOD and GPX activity comparably gave better results with increasing concentration of Cu (Figs. 3a, b and 4a, b). CAT, SOD and GPX seem to be important in controlling the excess of H_2O_2 that Cu can induce. The findings point out that there is a strong dose–response relationship regarding antioxidant enzyme activity in both the samples (leaf and root) with Cu concentration in the nutrient solution, and this phenomenon has also been reported in bean plants (Cuypers et al. 2002). Under Cu stress, only one CAT activity band was observed in both the tissue samples (Fig. 2c, d). The intensities of band patterns were varied in different concentration of Cu treatment. In case of leaf, the intensity of a band was increased at 50 and 25 μM of Cu in 7 and 14 days treated plants. Whereas in root, the intensity was maximum in 100 and 25 μM of Cu in 7 and 14 days treated plants respectively (Fig. 2c, d). The isoenzymic patterns for both leaves and roots are similar to those obtained by Vitoria et al. (2001) in a study of cadmium stress in *R. sativus* tissues. SOD activity also showed an increase in isoenzymes of SOD under excess concentration of Cu (Fig. 3c, d). However, the activity tends to decrease at longer exposure (14 days) at the highest Cu concentration. Several authors have reported an increase in SOD activity with Cu stress in *Morus alba* (Tewari et al. 2006) and *Elsholtzia splendens* (Peng et al. 2006). Similar results were also observed in GPX activity. With the increase in Cu concentration the number of isoenzymes increased, but after a specific concentration, the bands disappeared in both the tissue samples (Fig. 4c, d). Our results indicate an enhancement in the activity of GPX, suggesting that this enzyme serves as an intrinsic defense tool to resist Cu-induced oxidative damage in *Withania* plant. Induction in GPX activity has been documented under a variety of stressful conditions such as Cu (Khatun et al. 2008) and salinity excess (Behera et al. 2009). Khatun et al. (2008) also studied the toxicity of Cu on *W. somnifera* by taking nodal explants under *in vitro* condition and found that the leaf samples of Cu-treated plants decreased its CAT and SOD activity. But in our case, a contradictory result was obtained that the CAT and SOD activity is significantly increased up to 50 μM of Cu both in leaf and root samples.

The results presented in this work show that *W. somnifera* can cope well with moderate concentrations of Cu and at higher concentration this metal induces oxidative stress. The accumulation of Cu in this plant contributed to the formation of ROS, which confirmed via the antioxidative enzymatic defense mechanism by stimulating an increase in CAT, SOD and GPX activity. However, more information is needed at the sub-cellular and molecular levels in order to gain deeper insight into the mechanisms of Cu toxicity, as well as a relationship of the genes to these enzymes.

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