



Sesbania drummondii cell cultures: ICP-MS determination of the accumulation of Pb and Cu

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Abstract

Sesbania cell cultures grown in the presence of different concentrations of Pb (0–1000 mg/L) and Cu (0–500 mg/L) were assayed for growth, metal accumulations and activities of antioxidative enzymes: superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (GPX). These cultures tolerated Pb up to a concentration of 500 mg/L, registering a fresh weight growth of 500% in 3 weeks. At the same time, cultures registered a growth of 200% in 3 weeks at a Cu concentration of 100 mg/L showing less tolerance than Pb. However, *Sesbania* cells accumulated more Cu than Pb, as determined by ICP-MS, at all the treatments tested. Cu accumulation reached 3000 mg/kg (dry weight) at a Cu treatment of 100 mg/L, while Pb accumulation was only over 150 mg/kg (dry weight) at 500 mg Pb/L. Metal accumulations were positively correlated with induction of SOD and CAT activities in both the metal treatments. SOD activity of callus was 105 U/mg (fresh weight) at a Pb treatment of 500 mg/L and the corresponding Pb accumulation of 160 mg/kg (dry weight), while the activity rose to 300 U/mg (fresh weight) at a Cu treatment of 100 mg/L and the corresponding Cu accumulation of 3000 mg/kg (dry weight). The pattern of GPX activities was, however, different, particularly in Pb treatments where activities declined with increasing concentrations of Pb in the cells as well as growth medium. This study shows how *Sesbania* cells withstand heavy metal stress by induction of antioxidative enzyme activities. © 2005 Elsevier B.V. All rights reserved.

Keywords: *Sesbania drummondii* cell culture; ICP-MS; Pb, Cu accumulations

1. Introduction

Heavy metals like Cu, Cd, Ni, Pb, Zn are major environmental pollutants, potentially considered to be toxic, mutagenic and carcinogenic though a few of them are essential elements for vital metabolic processes [1]. The most pronounced effect of heavy metals on plant development is growth inhibition, which is inseparably connected with cell division. However, the mechanisms involved in those processes are still not completely understood [2]. The intoxication with pollutant metals induces oxidative stress because they are involved in various types of ROS (reactive oxygen species)-generating mechanisms. Transition metals such as Cu²⁺ and Fe³⁺ participate in the well-known Haber-Weiss cycle, producing ·OH from O₂⁻ and H₂O₂ [3]. Metals

without redox capacity such as Pb²⁺ and Cd²⁺ can enhance the pro-oxidant status by reducing the antioxidant glutathione (GSH) pool, activating calcium-dependent systems and affecting iron-mediated processes. These metals can also disrupt the photosynthetic electron chain, leading to the production of superoxide anion (O₂⁻) and singlet oxygen [O₂(¹Δ_g)] [3]. Irrespective of the production pathway, ROS are highly cytotoxic and their level within plant cells must be controlled by enzymatic and non-enzymatic antioxidant defense systems [4]. Therefore, modulation of antioxidant levels constitutes an important adaptive response to withstanding adverse conditions generated by heavy metal exposure.

Plants have developed a wide range of protective mechanisms that serve to remove ROS before they can damage sensitive parts of the cellular machinery [4–6]. The battery of antioxidant enzymes expressed by the plant cells includes: superoxide dismutases (SOD), catalase (CAT), guaiacol peroxidase (GPX) and ascorbate peroxidase

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(APX), etc. [7]. SOD, which catalyzes the disproportionation of O_2^- to O_2 and H_2O_2 has been called the cell's first line of defense against ROS. O_2^- is a precursor to several other highly reactive species and thus control over the steady state O_2^- concentration by SOD constitutes an important protective mechanism [8]. Hydrogen peroxide (H_2O_2) is scavenged directly by catalase (CAT), which converts it to water and molecular oxygen. Peroxidases also scavenge H_2O_2 indirectly by combining it with antioxidant compounds such as ascorbate and guaiacol [9,10].

There are a number of plant species that grow and accumulate heavy metals and thus find use in various phytoremediation strategies for decontamination of the environment [11–13]. *Sesbania drummondii*, a perennial large bushy plant with high biomass productivity and natural distribution in southern coastal plains of the United States, is one of such plant species. It demonstrates a unique potential of Pb accumulation in aerial parts from an aqueous solution [14]. When grown in Pb-contaminated soil (supplemented with chelators) in a greenhouse, this species showed a substantial movement of Pb from roots to shoot with appreciable amounts being accumulated in the aerial parts [15]. Though *Sesbania* and other plants tolerate toxic concentrations of Pb, the specific mechanisms of tolerance are not well understood. Using X-ray absorption spectroscopy (XAS), Sharma et al. [16] demonstrated the internalized speciation of Pb in roots and leaves of *Sesbania* seedlings where Pb was predominantly bound to sulfur compounds. This report also indicated that this plant used more than one mechanism to protect its cellular components from Pb toxicity. Therefore, there is a possibility that this plant uses antioxidative defense more effectively to prevent the oxidative insult that is produced by heavy metal accumulation.

Plants or cells in vitro have been shown to adapt to toxic concentrations of metal ions and natural or artificial selection can be effective in identifying tolerant genotypes from non-tolerant populations [17,18]. Several cell lines resistant to abiotic stress have been isolated [19–21]. Before Pb- or Cu-resistant *Sesbania* cell lines can be developed, the present investigation was undertaken to discern the metal tolerance potential and adaptive mechanisms in *Sesbania* cell cultures. In view of these facts, the objective of this study was to determine (i) the growth profile, (ii) metal accumulation pattern using ICP-MS in *Sesbania* cell cultures grown in high concentrations of Pb and Cu, and (iii) to correlate metal accumulations with the activity of antioxidative enzymes: SOD, CAT and GPX, which confer an adaptive advantage in resistance.

2. Materials and methods

2.1. Establishment of *Sesbania* suspension culture

Callus was induced from hypocotyls or cotyledonary leaf segments of *Sesbania* seedlings as described by

Cheepala et al. [22]. After 4–6 weeks of callus proliferation, 10 g of callus mass was homogenized aseptically in 50 ml of the Murashige and Skoog (MS) [23] medium, and used as a stock culture to initiate suspension cultures in 250 ml flasks. Equal volumes (5 ml) of the stock cultures were transferred to 100 ml of MS medium containing 2 mg/L BA (benzyladenine) and 1.5 mg/L NAA (naphthaleneacetic acid). Culture flasks were placed at a shaker (125 rpm) and incubated for 4–6 weeks at 25 ± 2 °C in 16 h photoperiod of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance.

2.2. Metal treatments and growth assay

Exponentially growing cells were filtered through three layers of sterile cheese cloth and resuspended in fresh growth medium at a concentration of 200 mg (wet weight) cells per milliliter. This inoculum suspension was continuously stirred while pipetting 1 ml aliquots onto the surface of 9-cm discs of Whatman No. 2 qualitative filter paper that had been placed on 25 ml of agar (0.8%) medium in 100 mm \times 25 mm plastic petri plates. The agar-growth medium (MS) contained 2 mg/L BA, 1.5 mg/L NAA and different concentrations of $Pb(NO_3)_2$ (0–1000 mg/L) or $CuSO_4 \cdot 5H_2O$ (0–500 mg/L) separately for the growth of cells under different metal treatments. Cultures were incubated at 25 ± 2 °C in 16 h photoperiod of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance. Fresh weight of culture was measured using Filter paper growth assay [24]. Culture growth was recorded at 1–3 weeks and expressed in % increase in fresh weight.

2.3. Estimation of cellular Pb and Cu

Cell clumps or calli harvested at 1–3 weeks (four replicates each treatment) were washed with 10 mM $CaCl_2$ to remove extracellular metals, rinsed with distilled water, and oven-dried at 70 °C for 2 day. Samples were weighed and placed in a 15 mL screw capped Teflon beaker. Three milliliters concentrated (16 N) HNO_3 was added to the sample and beaker was placed on a hot plate at a temperature of 100 °C overnight, then evaporated to dryness. The dried materials were allowed to cool and made up gravimetrically with 2% HNO_3 to a volume of 20 mL [14]. A VG Elemental Plasma Quad (model PQ2STE) ICP-MS was used for all data acquisition. Single element standard solutions (Inorganic Ventures, Lakewood, NJ) were utilized to prepare calibration and internal standard solutions. Analyses were performed using an external calibration procedure, and internal standards were included for matrix and instrumental drift corrections. For data reduction, the raw intensities were corrected for background counts, instrumental drift, matrix effects, and wherever applicable for molecular interferences. Procedural blanks were analyzed to check for any contribution from the reagents.

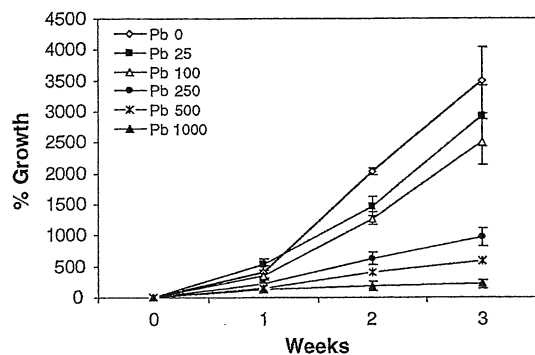


Fig. 1. Growth of *S. drummondii* cell cultures on the MS medium containing 0–1000 mg/L $\text{Pb}(\text{NO}_3)_2$ in 1–3 weeks. Fresh weight growth (expressed in percent) represents means \pm SE ($n=5$).

2.4. Statistical analysis

Means of at least five replicates per treatment were shown as data points for each parameter of study. Analysis of data was carried out by using SYSTAT (version 9.0 for Windows, Systat Software Richmond, USA). The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences ($P < 0.05$) among the treatment means and Tukey HSD post hoc test was performed to compare among the groups for significant differences.

2.5. Antioxidant enzyme activity assays

Calli from different treatments [0–500 mg/L $\text{Pb}(\text{NO}_3)_2$ and 0–100 mg/L CuSO_4] were harvested after 3 weeks and homogenized in cold phosphate buffer (pH 7.0). One ml of phosphate buffer was used per 100 mg callus fresh weight (FW). After homogenization in cold phosphate buffer, homogenates were centrifuged at $4000 \times g$ at 4°C for 10

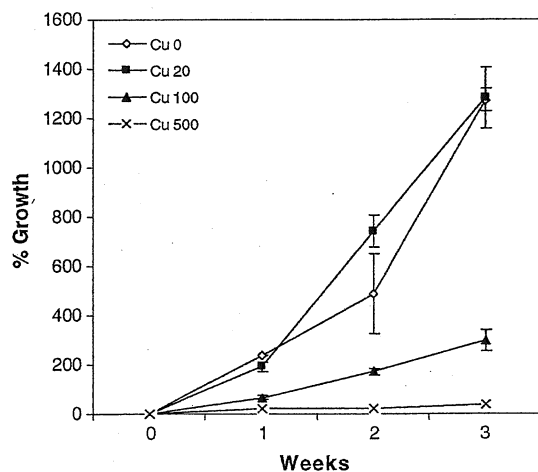


Fig. 2. Growth of *S. drummondii* cell cultures on the MS medium containing 0–500 mg/L CuSO_4 in 1–3 weeks. Fresh weight growth (expressed in percent) represents means \pm SE ($n=5$).

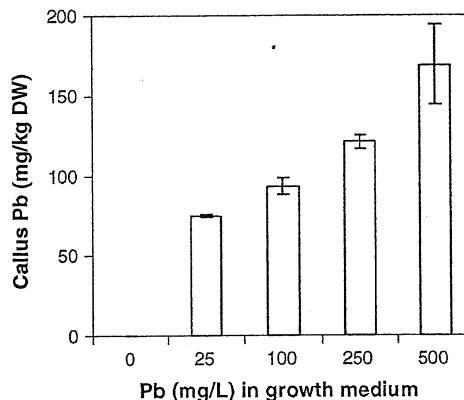


Fig. 3. Accumulations of Pb by *S. drummondii* callus (cells) grown in the presence of 0–1000 mg/L $\text{Pb}(\text{NO}_3)_2$. Values represent means \pm SE ($n=5$).

min to remove plant debris. Unless stated otherwise, the supernatant was then diluted fivefold in phosphate buffer and assayed for antioxidant enzyme activity.

2.5.1. Superoxide dismutase (SOD)

Activity of SOD was tested using the WST SOD assay kit from Dojindo Molecular Technologies (Gaithersburg, Maryland, USA), as described by Ukeda et al. [25]. One unit was defined as the amount required to inhibit the reduction of WST-1 to WST-1 formazan in the presence of superoxide by 50%.

2.5.2. Catalase (CAT)

CAT activity was assayed using the method described by Aebi [26]. A reaction mixture consisting of 666 μl supernatant and 334 μl of 73 mM H_2O_2 (Fisher Scientific) was then assayed for 3 min at 240 nm. Activity was measured as disappearance of H_2O_2 . One unit of enzyme activity was defined as a decrease in absorbance of 0.001 min^{-1} at 240 nm.

2.5.3. Guaiacol Peroxidase (GPX)

GPX activity was measured using the method described by the United States Environmental Protection Agency [27].

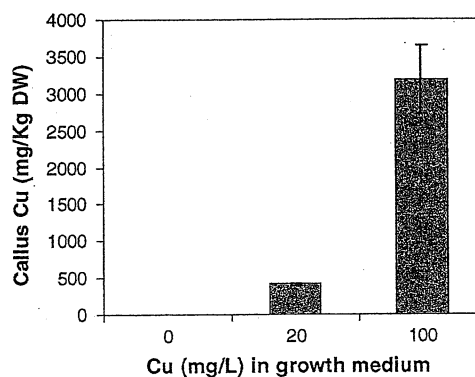


Fig. 4. Accumulations of Cu by *S. drummondii* callus (cells) grown in the presence of 0–100 mg/L CuSO_4 . Values represent means \pm SE ($n=5$).

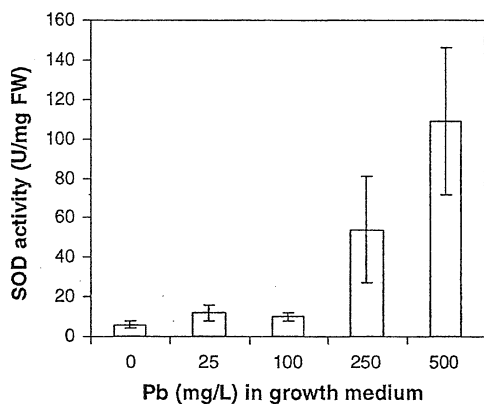


Fig. 5. Superoxide dismutase activity (U/mg fresh weight) in *S. drummondii* cell cultures exposed to 0–1000 mg/L $\text{Pb}(\text{NO}_3)_2$ for 3 weeks. Values represent means \pm SE ($n=5$).

A reaction mixture consisting of supernatant (100 μl), 17 mM H_2O_2 (450 μl) (Fisher Scientific), and 2% guaiacol (450 μl) (Fisher Scientific) was then assayed for 3 min at 510 nm. Activity was measured as appearance of tetra-guaiacol. One unit of enzyme activity was defined as an increase in absorbance of 0.001 min^{-1} at 510 nm.

3. Results

3.1. Growth of *Sesbania* cells

Sesbania cell cultures grew and proliferated into callus mass over time on increasing concentrations of $\text{Pb}(\text{NO}_3)_2$ in the medium (Fig. 1). Twenty- to thirty-fold growth was recorded at lower concentrations of this metal (25 and 100 mg/L), which is comparable to the control growth. Significant growth inhibition was observed when cells were subjected to a Pb concentration of 250 mg/L. The growth was severely affected at and above 500 mg/L $\text{Pb}(\text{NO}_3)_2$, but gain in callus biomass after 3 weeks was four- and twofolds at 500 and 1000 mg Pb/L, respectively (Fig. 1). Growth pattern in *Sesbania*

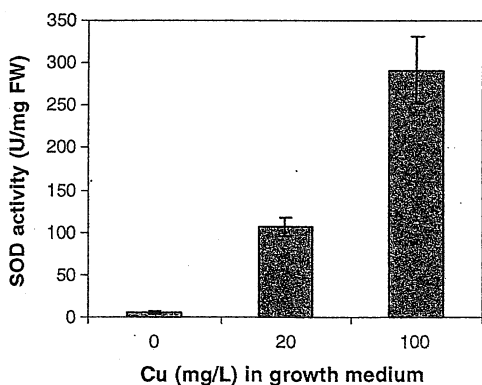


Fig. 6. Superoxide dismutase activity (U/mg fresh weight) in *S. drummondii* cell cultures exposed to 0–100 mg/L CuSO_4 for 3 weeks. Values represent means \pm SE ($n=5$).

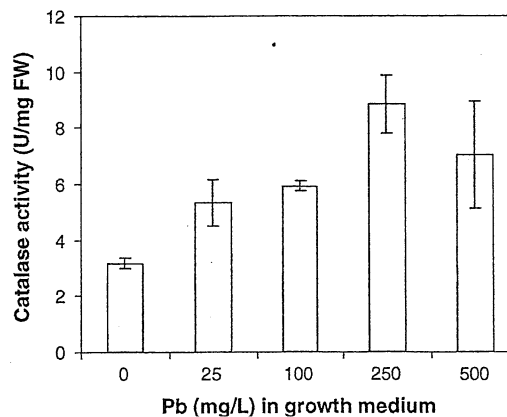


Fig. 7. Catalase activity (U/mg fresh weight) in *S. drummondii* cell cultures exposed to 0–1000 mg/L $\text{Pb}(\text{NO}_3)_2$ for 3 weeks. Values represent means \pm SE ($n=5$).

cells was different at Cu treatments (Fig. 2). While lower concentration of Cu (20 mg/L) favored growth, higher concentrations (100 and 500 mg/L) were inhibitory. However, at a treatment of 100 mg/L Cu, cells registered >200% growth (after 3 weeks) and formed good callus mass. Gain in biomass was negligible at 500 mg/L Cu (Fig. 2).

3.2. Metal accumulations in callus

Fig. 3 shows accumulation of Pb in the callus mass on increasing concentrations of Pb. Increase in accumulation in cells was directly proportional to the concentration of Pb present in the medium. But a dramatic increase in accumulation (>6000 mg/kg tissue DW) was observed at a concentration of 1000 mg/L (data not presented). Accumulation of Cu was also dependent on the concentration of CuSO_4 present in the medium (Fig. 4). *Sesbania* cells accumulated higher amounts of Cu than Pb at both the concentrations of Cu tested. Cu accumulation in the callus was much higher (about 3000 mg/kg) at a treatment of 100 mg Cu/L. Accumulation at 500 mg/L could not be

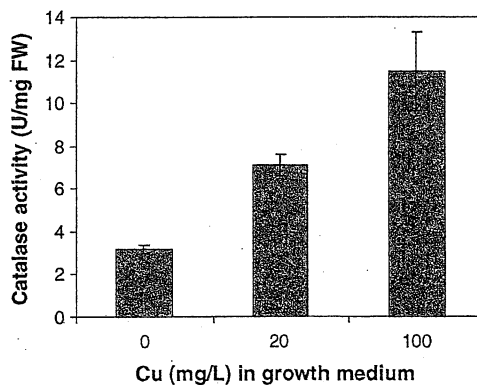


Fig. 8. Catalase activity (U/mg fresh weight) in *S. drummondii* cell cultures exposed to 0–100 mg/L CuSO_4 for 3 weeks. Values represent means \pm SE ($n=5$).

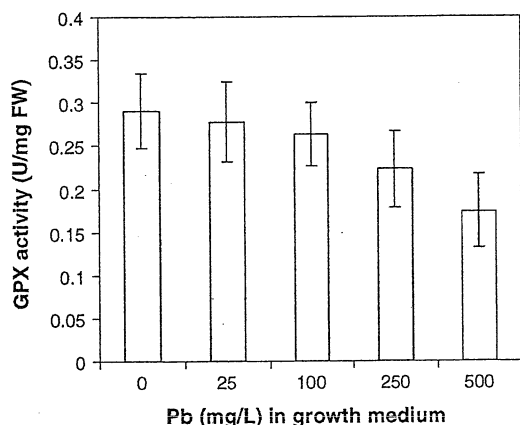


Fig. 9. Guaiacol peroxidase activity (U/mg fresh weight) in *S. drummondii* cell cultures exposed to 0–1000 mg/L $\text{Pb}(\text{NO}_3)_2$ for 3 weeks. Values represent means \pm SE ($n=5$).

recorded as the amount of dried tissue was not sufficient for analysis.

3.3. Antioxidant enzyme activities in callus

SOD activity: A direct correlation was noticed between the activity of SOD and $\text{Pb}(\text{NO}_3)_2$ concentrations in medium or callus (Fig. 5). The activity was more or less similar to the control value up to the treatment of 100 mg Pb/L. The activity sharply rose to 60 and 115 U/mg (FW) at the treatments of 250 and 500 mg Pb/L, respectively. A similar trend was recorded in Cu treatments, where SOD activity was directly proportional to the concentration of CuSO_4 in medium or callus (Fig. 6). The activity sharply rose from 100 U/mg (FW) to 300 U/mg (FW) when the concentration of Cu in the medium increased from 20 mg/L to 100 mg/L.

CAT activity: Fig. 7 demonstrates the relationship between the catalase activity and concentrations of Pb in medium or callus. The activity increased gradually with the increase of Pb concentration in medium, and rose finally threefolds at 250 mg/kg Pb followed by a decline at 500 mg/L. Catalase activities of *Sesbania* cells followed a similar trend on Cu treatments and a fourfold increase in CAT activity was recorded (Fig. 8) at 100 mg/L.

GPX activity: The pattern of GPX activities was observed different than SOD and CAT activities in *Sesbania* cells (Fig. 9). GPX activity in cell mass either remained unchanged statistically or decreased, particularly at higher concentrations of Pb treatments (Fig. 9). However, in case of Cu treatments GPX activities increased significantly at 20 and 100 mg/L (Fig. 10).

4. Discussion

This study demonstrates that *S. drummondii* cell cultures not only tolerate but grow profusely on inhibitory

concentrations of Pb and Cu. Though growth was inhibited significantly (at higher concentrations) as compared to the control, the increase in biomass recorded at a treatment of 100 mg $\text{Pb}(\text{NO}_3)_2/\text{L}$ was enormous, about 20-folds after 3 weeks. Studies on mechanisms of Pb toxicity suggest that Pb^{2+} also binds to nucleic acids and causes aggregation and condensation of chromatin, as well as stabilization of DNA double helix inhibiting the processes of replication, transcription and ultimately the cell division [28]. It appears that this plant has mechanisms to minimize the cytological effects of Pb^{2+} by inactivating or excluding the available ions from within the cell. The previous X-ray absorption studies [16] on *Sesbania* seedlings showing internalized binding of Pb^{2+} with sulfur compounds and vacuolar deposition of Pb give credence to this hypothesis. The effect of higher concentrations of Cu on cell cultures of this plant was more severe, though cells increased in biomass >2-folds after 3 weeks in presence of Cu (100 mg/L). The pattern of cell growth in the presence of Cu was comparable to the tobacco BY-2 suspension cultures [29], where cells showed profuse growth in presence of high concentration of Cu (2.5 g/L).

Similar to the previous report of Pb accumulation by whole plants [14], cell cultures of this species also demonstrate Pb accumulations in the concentration-dependent manner. Accumulation particularly at the highest concentration of Pb (1000 mg/L) was enormous (>6000 mg Pb/kg DW) and this affected callus morphology and growth dramatically. Likewise, accumulations of Cu were many hundred-folds as compared to the control (without Cu treatment) at the Cu concentrations of 20 and 100 mg/L in media.

Accumulations of metals in *Sesbania* cells were accompanied by concomitant induction of activities in antioxidative enzymes such as SOD, CAT and peroxidase (GPX). Six- and twelve-fold rise in SOD activity at 250 and 500 mg Pb/L, respectively, correlates well to the increased accumulations of Pb at these concentrations. Similar increase in SOD activity in pea root cells was

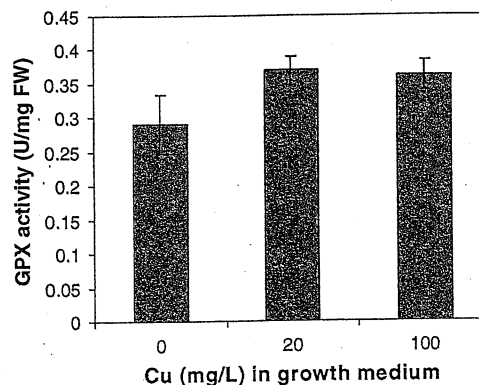


Fig. 10. Guaiacol peroxidase activity (U/mg fresh weight) in *S. drummondii* cell cultures exposed to 0–100 mg/L CuSO_4 for 3 weeks. Values represent means \pm SE ($n=5$).

reported at a Pb concentration of 104 or 207 mg/L [10]. Unlike Pb treatments, Cu treatments caused a dramatic induction of SOD activities in cell cultures of this plant. Twenty- and sixty-fold increase in its activity, compared to the control, was noticed at 20 and 100 mg/L Cu treatments. The correlation between the SOD activity level and the heavy metal stress effect depends on the plant species and the type of the metal. In other leguminous plants like soybean, pea and yellow lupine many researchers observed a substantial increase in the activity of this enzyme after 2 days of Pb²⁺, Cu²⁺, Al³⁺ and Mn²⁺ treatments [30–32]. Sunflower callus, adapted to grow in high concentrations of Cr, had the SOD activity [20] comparable to that of *Sesbania* callus cultivated in presence of Cu.

It seems likely that enormous amounts of Cu accumulated in these cells/calli induce activation of SOD at an accelerated rate. Toxic metal ions not only influence total SOD activity but also cause changes in SOD isoforms pattern. Three major SOD isoforms have been described in eukaryotic photosynthetic organisms (4): a Cu,ZnSOD located in the thylakoid membrane and cytosol of higher plants, a MnSOD isoform found within mitochondria; and an FeSO₄ isoform in the chloroplast stroma. Interestingly, SOD is induced by its substrate [33] and thus activation of specific SOD isoforms can serve as an indicator of the cell compartment experiencing pollutant-induced O₂^{•-} levels. In this experiment, Cu-induced multi-fold activation suggests the preponderance of Cu,ZnSOD expressed either in the cytosol or thylakoid membranes to ward off Cu²⁺ toxicity. Similar augmentation in Cu,ZnSOD activity was observed in tobacco BY-2 cell culture on exposure to 100 mM Cu [29].

Activity of catalase, another important enzymatic component of the antioxidative defense system, was also induced in response to Pb or Cu accumulations in these cells. The three- to fourfold increase at higher concentrations of Pb or Cu is comparable with the reported CAT activity in pea root cells grown in Pb [10]. Results in *Sesbania* cultures are also compatible to CAT activities reported in the adapted calli of sunflower, grown under Cd, Al and Cr treatments [20]. However, activities decreased in response to the growth inhibiting concentrations of Cu and Cd in tobacco cell culture [29], and soybean suspension culture [34], respectively. Findings in this experiment also support that the pattern of CAT expression, alike SOD, depends on the plant species and heavy metal type.

In this experiment GPX activity was measured as an indicator of nonspecific peroxidases. Unlike SOD or CAT, GPX activities in these cultures either remained unchanged or decreased at higher concentrations of Pb. The pattern was a little different in Cu treatments, where a moderate increase in activity (20%) was observed in calli cultivated at 20 or 100 mg/L Cu. Application of 50 μM Pb-EDTA led to an induction of 229% activity of this enzyme in *Phaseolus* sp. [35], while activity was decreased by Cd

and Cr treatments and remained similar to the control with Al in case of sunflower cell culture [20]. Variable patterns of GPX activity have been noticed in plants.

The results of this work clearly demonstrate a direct relationship between toxic metal accumulations and activities of antioxidative enzymes. The hypothesis that antioxidative enzymes are important components in preventing the oxidative stress in plants is based on the fact that the activity of one or more of these enzymes is generally increased in plants when exposed to stressful conditions and this elevated activity correlated to increased stress tolerance [35]. Overexpression of genes encoding these enzymes in several transgenic plant species conferring protection against free radicals has also been demonstrated [7,36]. In studies on *Sesbania* cell cultures, Pb or Cu treatments resulted in a significant rise in the activities of SOD and CAT ($P < 0.05$), which can be considered as a circumstantial evidence for enhanced production of free radicals under Pb or Cu stress. The differential activities (higher in case of Cu treatments) of these enzymes support this view, as the nature of stress causing metals varies, one with (Cu), another without (Pb) redox capacity.

Although the antioxidative defense system was undoubtedly involved in the strategy used by *Sesbania* cells to survive under high heavy metals concentration, the variation in the degree of response (with respect to different enzymes) demonstrates that multiple mechanisms rather than a single mechanism may be responsible for the adaptation of the cells to resist metal stress. Therefore, Parker and Pedler [37] proposed that a multifaceted model of tolerance is needed to explain Al tolerance in plant tissues. A deeper insight into the identification of the multiple isoforms of the enzymes or their localization inside the cells is necessary to elucidate the precise role of the antioxidative components in the resistance process. Oxidation state and coordination environment of elements also influence the oxidative stress in plants [16]. For example, arsenate (As^v), chromate (Cr^{vi}) and selenate are more toxic to plants and thus some hyperaccumulator plants preferentially reduce these toxic ions to less harmful ones as detoxification mechanisms. In this background, it is speculated that other sources of copper or lead may also affect *Sesbania* cells differently, triggering a differential pattern of antioxidative responses.

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References

- [1] S. Rama Devi, M.N.V. Prasad, *Plant Sci.* 138 (1998) 157.

- [2] K.-J. Dietz, M. Baier, U. Kramer, *Heavy Metal Stress in Plants: From Molecules to Ecosystems*, Springer-Verlag, Berlin, 1999, p. 73.
- [3] E. Pinto, T.C.S. Sigaud-kutner, M.A.S. Leitao, O.K. Okamoto, D. Morse, P. Colepiccolo, *J. Phycol.* 39 (2003) 1008.
- [4] K. Asada, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50 (1999) 601.
- [5] O.K. Okamoto, D.L. Robertson, T.F. Fagan, J.W. Hastings, P. Colepiccolo, *J. Biol. Chem.* 276 (2001) 19989.
- [6] R.G. Alscher, N. Erturk, L.S. Heath, *J. Exp. Bot.* 53 (2003) 1131.
- [7] C.H. Foyer, P. Descourvieres, K.J. Kunert, *Plant Cell Environ.* 17 (1994) 507.
- [8] I. Fridovich, *J. Biol. Chem.* 250 (1975) 18515.
- [9] M. Gupta, A. Cuypers, J. Vangronsveld, H. Clijsters, *Physiol. Plant.* 106 (1999) 262.
- [10] A. Malecka, W. Jarmuszkiwicz, B. Tomaszewska, *Acta Biochim. Pol.* 48 (2001) 687.
- [11] J.W. Huang, S.D. Cunningham, *New Phytol.* 134 (1996) 75.
- [12] M.J. Blaylock, D.E. Salt, S. Dushenkov, O. Zakharova, C. Gussman, Y. Kapulnik, B.D. Ensley, I. Raskin, *Environ. Sci. Technol.* 31 (1997) 860.
- [13] J.W. Huang, J. Chen, W.R. Berti, S.D. Cunningham, *Environ. Sci. Technol.* 31 (1997) 800.
- [14] S.V. Sahi, N.L. Bryant, N.C. Sharma, S.R. Singh, *Environ. Sci. Technol.* 35 (2002) 4676.
- [15] A.T. Thomas, M.S. Thesis, Western Kentucky University, Bowling Green, USA, 2004.
- [16] N.C. Sharma, J.L. Gardea-Torresdey, J. Parsons, S.V. Sahi, *Environ. Toxicol. Chem.* 23 (2004) 134.
- [17] P.J. Jackson, E.J. Roth, P.R. McClur, C.M. Narango, *Plant Physiol.* 75 (1984) 914.
- [18] R.M. Krotz, B.P. Evangelou, G.J. Wagner, *Plant Physiol.* 91 (1989) 780.
- [19] J.C. Steffens, D.F. Hunt, G.B. Williams, *J. Biol. Chem.* 261 (1986) 13879.
- [20] S. Gallego, M. Benavides, M. Tomaro, *Plant Growth Regul.* 36 (2002) 267.
- [21] S.A. Watmough, T.C. Hutchinson, *Can. J. For. Res.* 27 (1997) 693.
- [22] S.B. Cheepala, N.C. Sharma, S.V. Sahi, *Biol. Plant.* 48 (2004) 13.
- [23] T. Murashige, F. Skoog, *Physiol. Plant.* 15 (1962) 473.
- [24] R.B. Horsch, J. King, G.E. Jones, *Can. J. Bot.* 58 (1980) 2402.
- [25] H. Ukeda, T. Shmamura, M. Tsubouchi, Y. Harada, Y. Nakai, Y. Sawamura, *Anal. Sci.* 18 (2002) 1151.
- [26] H.E. Aebi, Catalase, in: H.U. Bergmeyer (Ed.), *Methods of Enzymatic Analysis*, 3rd ed., Academic Press, New York, 1983, p. 273.
- [27] United States Environmental Protection Agency, Plant peroxidase activity determination, SOP# 2035, 11/28/94.
- [28] B.L. Valle, D.D. Ulmer, *Annu. Rev. Biochem.* 41 (1972) 91.
- [29] P. Bueno, A. Piqueras, *Plant Growth Regul.* 36 (2002) 161.
- [30] I. Cakmak, W.J. Horst, *Physiol. Plant.* 8 (1991) 463.
- [31] L.A. del Rio, L.M. Sandalio, J. Yanez, M. Gomez, *J. Inorg. Biochem.* 24 (1985) 25.
- [32] R. Przymusinski, R. Rucinska, E.A. Gwozdz, *Environ. Exp. Bot.* 35 (1995) 485.
- [33] R.G. Allen, M. Tresini, *Free Radic. Biol. Med.* 28 (2000) 463.
- [34] R. Sobkowiak, K. Rymer, R. Rucinska, J. Deckert, *Acta Biochim. Pol.* 51 (2004) 219.
- [35] W. Geebelen, J. Vangronsveld, D.C. Adriano, L.C. Van Poucke, H. Clijsters, *Physiol. Plant.* 115 (2002) 377.
- [36] R.D. Allen, *Plant Physiol.* 107 (1995) 1049.
- [37] D.R. Parker, J.F. Pedler, *Planta* 205 (1998) 389.