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Interactive effects of lead, copper, nickel and zinc on growth, metal uptake and antioxidative metabolism of *Sesbania drummondii*

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ABSTRACT

Sesbania drummondii seedlings were grown in a medium to which lead (Pb), copper (Cu), nickel (Ni) and zinc (Zn) were added singly and in combinations in order to assess the effects of metal interactions on seedling growth, metal accumulation and anti-oxidative system. *S. drummondii* growth was significantly inhibited with metal treatments. *S. drummondii* accumulated substantially higher concentrations of metals in the roots than shoots. The uptake of metals followed the order Pb > Cu > Zn > Ni in roots and Pb > Zn > Cu > Ni in shoots. In addition, uptake of a single metal by *S. drummondii* was affected by the presence of a second metal, suggesting an antagonistic effect or competition between metals at the plant uptake site. A significant increase in both enzymatic [superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR)] and non-enzymatic (glutathione) antioxidant was observed in the *S. drummondii* seedlings exposed to different metal treatments. The enhancement in enzyme activities followed the order of Cu > Ni > Pb > Zn. However, compared to the effect of individual metal, metals in combination increased the enzyme activities to varying degrees.

1. Introduction

Compound pollution of trace elements is a common phenomenon in nature where additive, synergistic, and antagonistic effects can occur [1]. It occurs not only in mining and sewage irrigation areas, but also in suburban vegetable plots caused by atmospheric deposition or additives [2]. Heavy metals like copper (Cu), nickel (Ni) and zinc (Zn) are essential micronutrients for plants, but in excess all these metals are harmful to humans, animals and plants; as are the non essential metals Pb, Cd and Hg [3]. The primary sources of these metals are the burning of fossil fuels, the mining and smelting of metalliferous ores, municipal wastes, fertilizers, pesticides and sewage [4].

Heavy metals are known to induce oxidative stress by generating high concentrations of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). These species react very rapidly with lipids, nucleic acids, pigments and proteins and cause lipid peroxidation, membrane damage and inactivation of enzymes, thus affecting cell viability [5]. Plants cope with oxidative stress by using antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and glutathione reductase (GR) and the low molecular weight antioxidants like cysteine, non-protein thiols, ascorbic acid and glutathione [6]. The enzyme SOD catalyzes the $O_2^{\bullet -}$ to H_2O_2 and oxygen. However, high concentration of H_2O_2 is also toxic to cells and has to be further detoxified by catalase and peroxidases to water and oxygen. Glutathione, cysteine and ascorbic acid can directly interact with and detoxify oxygen free radicals [7].

Several technologies are available to remediate soils that are contaminated by heavy metals. However, many of these technologies (for example, excavation of contaminated material and chemical/physical treatment) are extremely costly or do not achieve a long term solution [8,9]. Phytoremediation is defined as the use of plants to remove or sequester hazardous contaminants from various media such as soil, water and air [10]. This technique has become a tangible alternative to traditional methodologies. More than four hundred metal hyperaccumulator plants are known, they can accumulate high concentrations of metals into their aboveground biomass [4]. Hyperaccumulators are defined as plants that can accumulate >1000 mg kg^{-1} of Cu, Co, Cr, Ni and Pb, and >10,000 mg kg^{-1} of Mn and Zn [11]. However, many of the hyperaccumulator plants are of small biomass and have a slow growth rate, thus limiting their usefulness for phytoextraction [12]. In addition, very few plants are known that can accumulate more than one metal. Therefore, researchers are searching new plant species that could potentially be used to remediate different metals from contaminated sites. Furthermore, compared to single metal

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studies, there have been relatively fewer reports on the interactive effects of heavy metals on plants [13–17]. *Sesbania drummondii* is a perennial shrub which is distributed in southern coastal areas of the United States. It has been studied extensively in relation to the accumulation of Pb, Hg and Cu [18–21]. However, the effect of combinations of metals (Pb, Cu, Ni and Zn) on growth, metal accumulation and physiology of *S. drummondii* has not been studied yet. Therefore, the present study was undertaken to determine the Pb, Cu, Ni and Zn accumulation capability of *S. drummondii* and to examine the possible interactions between these metals at roots and shoots level. In addition, this study also reports the involvement of various antioxidants (enzymatic and non-enzymatic) in the tolerance against Pb, Cu, Ni and Zn induced stress, individually and in combinations. The results of this study should be helpful to determine the potential application of *S. drummondii* to remediate different metals from contaminated sites and to elucidate the biochemical detoxification mechanisms against Pb, Cu, Ni and Zn-induced stress.

2. Experimental

2.1. Plant growth and metal treatment

The seeds of *S. drummondii* were scarified in 85% H_2SO_4 for 35 min, rinsed in running water for 1 h and then finally rinsed in deionized (DI) water for 5 min. Scarified seeds were germinated and grown in pro-mix (Premier Horticulture INC., Quakertown, PA, USA). Two-week-old seedlings were first acclimatized in Hoagland's solution (half strength) for 3 days, then seedlings were transferred in the medium containing the following treatments: (1) Pb, (2) Cu, (3) Ni, (4) Zn, (5) Pb + Cu, (6) Pb + Ni, (7) Pb + Zn, (8) Cu + Ni, (9) Cu + Zn, (10) Zn + Ni, and (11) Pb + Cu + Ni + Zn. Seedlings without metal treatment served as control. A known phytotoxic levels of all four metals were used in the treatments [22]. All four metals were added to the medium as $Pb(NO_3)_2$, $CuSO_4{\cdot}5H_2O$, $Ni(NO_3)_2$, and $ZnSO_4{\cdot}7H_2O$ at a concentration of Pb, 250 mg L^{-1}; Cu, 100 mg L^{-1}; Ni, 100 mg L^{-1}; and Zn, 300 mg L^{-1}, respectively. The pH of the medium was adjusted to 5.6. Four replicates per treatment were prepared. The seedlings were grown at 25 °C using 16/8 hour light/dark cycle in a growth chamber under 200 μmol m^{-2} s^{-1} fluorescent light for 10 days. The metal solutions were changed after every 48 h to prevent depletion of nutrients. Seedlings were harvested after 10 days of treatment for estimation of biomass, metals content, antioxidative enzyme activities and glutathione.

2.2. Metal accumulation

Harvested seedlings were thoroughly washed with 5 mM EDTA (ethylenediaminetetraacetic acid) and DI water for desorption of surface bound metals. The shoots and roots were then separated and oven dried (68 ± 2 °C) to constant weight. The dried tissues were weighed and transferred to digestion tubes. One milliliter of concentrated (16 N) HNO_3 was added to the samples and heated at a temperature of 100 °C until the samples were completely clear. The digested samples were transferred to clean tubes and diluted to 10 ml with DI water. The analyses of Pb, Cu, Ni and Zn were carried out using inductively coupled plasma spectroscopy (ICP).

2.3. Biomass accumulation

The seedlings were washed with DI water and gently dried on blotting paper. Seedlings were wrapped in paper sheet and dried at 68 ± 2 °C until no weight changes could be detected. Thereafter, their dry weights were determined and expressed in mg per seedling.

2.4. Photosynthetic activities

The seedlings were analyzed for photosynthetic activities by measuring chlorophyll a fluorescence parameters. This was performed by using the Handy-PEA instrument (Hansatech Instruments, UK). Fully expanded intact leaves were used to determine chlorophyll a fluorescence. Leaves were dark adapted for 30 min with a Hansatech clip and then given a 1 s pulse of red light. The following fluorescent parameters were measured: F_o, the minimum chlorophyll a fluorescence after the dark-adaptation, and F_m, the maximum fluorescence after the pulse of red light. From these two measurements the F_v (the variable fluorescence calculated as the difference between the minimal and maximal fluorescence), F_v/F_m (the ratio of variable to maximal fluorescence) and F_v/F_o (the ratio of variable to minimal fluorescence) values were determined.

2.5. Antioxidative enzymes

2.5.1. Extraction of enzymes

Fresh seedlings (2.0 g) were homogenized in a prechilled mortar pestle under ice-cold conditions in 6.0 ml of extraction buffer containing 50 mM phosphate buffer (pH 7.5), 0.5% Triton X-100, 1% polyvinylpyrrolidone (PVP), and 1 mM EDTA. The homogenate was filtered through cheese cloth and centrifuged at 15,000 × *g* for 20 min at 4 °C. The supernatant was used for measuring SOD, APX and GR activities. Protein content in the supernatant was estimated according to Bradford using bovine serum albumin as a standard [23].

2.5.2. Assay of superoxide dismutase (EC1.15.1.1)

SOD activity was measured using nitroblue tetrazolium (NBT) method by measuring the photoreduction of NBT at 560 nm [24]. The reaction mixture (1.5 ml) contained 100 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 2.25 mM NBT, 60 μM riboflavin and enzyme extract. After mixing, the contents in the cuvette were illuminated for 10 min. A tube with enzyme extract was kept in the dark serving as a blank, while the control tube contained no enzyme extract and was kept in the light. The absorbance was measured at 560 nm against a blank using UV–vis spectrophotometer (Model Ultrospec 3000, Pharmacia Biotech, USA). NBT reduction in the light was measured in the presence and absence of enzyme extract. SOD activity is presented as absorbance of control minus absorbance of sample, giving the total inhibition. One unit of activity is the amount of enzyme required to inhibit photoreduction of NBT by 50% and was expressed in units of the enzyme (mg^{-1} protein h^{-1}).

2.5.3. Assay of ascorbate peroxidase (EC1.11.1.11)

The APX activity was determined by the method of Nakano and Asada [25]. The reaction mixture (1 ml) contained enzyme extract, 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate and 0.3 mM H_2O_2. The oxidation of ascorbic acid was measured by the decrease in absorbance at 290 nm for 3 min using a spectrophotometer. The enzyme activity was calculated using the extinction coefficient 2.8 mM^{-1} cm^{-1} and expressed in enzyme units (mg protein)$^{-1}$. One unit of enzyme is the amount necessary to decompose 1 μmol of substrate per min at 25 °C.

2.5.4. Assay of glutathione reductase (EC1.6.4.2)

The activity of GR was measured by monitoring the glutathione-dependant oxidation of NADPH at 340 nm described by Rao et al. [26]. The reaction mixture (1 ml) contained 100 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA, 0.2 mM NADPH, 0.5 mM oxidized glutathione and enzyme extract. The reaction was allowed to run for 3 min and was measured using a spectrophotometer. The enzyme activity was calculated using extinction coefficient

Table 1
Bioaccumulation of different metals (Pb, Cu, Ni and Zn) in the shoots and roots of *S. drummondii* seedlings grown in different metals, separately and in combinations.

Treatments	Concentrations of metal in shoots (mg kg^{-1} dw)				Concentrations of metal in roots (mg kg^{-1} dw)			
	Pb	Cu	Ni	Zn	Pb	Cu	Ni	Zn
Control	19.5 ± 3.2^{a}	33.2 ± 5.5^{a}	24 ± 4.0^{a}	37 ± 7.5^{a}	40.8 ± 6.9^{a}	34.8 ± 6.6^{a}	72 ± 6.1^{a}	586 ± 20^{a}
Pb	1268 ± 46^{b}				58590 ± 4000^{b}			
Cu		445 ± 36^{b}				14040 ± 866^{b}		
Ni			340 ± 27^{b}				2104 ± 57^{b}	
Zn				1180 ± 66^{b}				10360 ± 558^{b}
Pb + Cu	1386 ± 47^{b}	276 ± 21^{c}			64790 ± 2200^{b}	10800 ± 602^{c}		
Pb + Ni	1305 ± 46^{b}		197 ± 20^{c}		60010 ± 2309^{b}		697 ± 54^{c}	
Pb + Zn	1614 ± 52^{c}			1873 ± 43^{c}	62200 ± 3464^{b}			5602 ± 230^{c}
Cu + Ni		231 ± 24^{c}	391 ± 34^{b}			15620 ± 450^{b}	1204 ± 60^{d}	
Cu + Zn		499 ± 31^{b}		1300 ± 72^{b}		12920 ± 487^{db}		8000 ± 405^{d}
Zn + Ni			403 ± 41^{b}	896 ± 39^{d}			837 ± 63^{c}	10240 ± 552^{b}
Pb + Cu + Ni + Zn	1015 ± 30^{d}	354 ± 35^{db}	109 ± 14^{d}	780 ± 29^{e}	32680 ± 5201^{c}	9835 ± 640^{c}	481 ± 21^{e}	8750 ± 463^{d}

Values are the mean of four replicates and within each column, those not followed by the same letter are significantly different ($P < 0.05$).

6.2 mM^{-1} cm^{-1} and expressed in enzyme units (mg protein)$^{-1}$. One unit of enzyme is the amount necessary to decompose 1 μmol of NADPH per min at 25 °C.

2.6. Estimation of glutathione content

One gram fresh seedlings were homogenized in 3.0 ml of 5% sulfosalicylic acid with mortar and pestle. The homogenate was centrifuged at 15,000 × *g* for 15 min and the resulting supernatant was used for glutathione measurement following the method of Anderson [27]. Supernatant (0.5 ml) was taken in a microfuge tube, to which 0.5 ml reaction buffer [0.1 M phosphate buffer (pH 7.0), 0.5 mM EDTA] and 50 μl of 3 mM 5,5′dithio-bis-(2-nitrobenzoic acid) (DTNB) were added. Absorbance for determination of GSH was read after 5 min at 412 nm using a spectrophotometer. To the same tube, 100 μl of NADPH (0.4 mM) and 2 μl GR was added to determine total glutathione; the reaction was allowed to run for 20 min. The amount of GSSG (oxidized) was calculated by subtracting GSH (reduced) from total glutathione concentrations. A standard curve was prepared from varying concentrations of reduced glutathione.

2.7. Statistical analysis

The mean values (±S.E.) are given in all the tables and figures. The data was analyzed statistically using SYSTAT (version 11 for windows, Systat software Inc., Richmond, CA). Significant differences among treatments were analyzed by one-way ANOVA, taking $P < 0.05$ as significant level, and Tukey-HSD post hoc test was conducted to pair wise comparisons between treatments.

3. Results

3.1. Metal concentrations in plant tissues

The concentrations of metals (Pb, Cu, Ni and Zn) in the roots and shoots of *S. drummondii* seedlings grown at different treatments (Pb, Cu, Ni, Zn, Pb + Cu, Pb + Ni, Pb + Zn, Cu + Ni, Cu + Zn, Zn + Ni and Pb + Cu + Ni + Zn) are shown in Table 1. The results show that the metal contents in the plant tissues varied among metals in different combinations. Accumulation of all the metals was substantially higher in roots than in shoots. *S. drummondii* accumulated significantly ($P < 0.05$) higher Pb in its roots as well as in shoots compared to other metals (Cu, Ni and Zn). The metal concentrations followed the order Pb > Cu > Zn > Ni in roots and Pb > Zn > Cu > Ni in shoots. For all different combinations of metal accumulation studied with *S. drummondii* seedlings, bioaccumulation of a single metal in the roots as well in the shoots was affected by the presence of a second metal, resulting in the inhibition or increase in the bioaccumulation of one metal over other (Table 1).

3.2. Effect of different metal treatments on plant growth

Plant biomass is a good indicator for the overall health of *S. drummondii* growing in the presence of heavy metals. Growth of *S. drummondii* seedlings was significantly ($P < 0.05$) inhibited with metal treatments (Fig. 1). The level of inhibition depended on metal types and their combinations. However, we have not noticed any toxicity symptoms like necrosis in the plant. Among the four metals tested in this study, Cu was the most toxic followed by Ni > Zn > Pb. Among the binary mixtures of metals, Ni + Zn were most toxic. Furthermore, maximum inhibition in *S. drummondii* growth was noticed in the mixture of all metals (Pb + Cu + Ni + Zn). Compared to the control (no metal), seedling growth significantly ($P < 0.05$) reduced by 21.0%, 46.3%, 31.5%, 25.2%, 45.7%, 41.0%, 36.3%, 42.1%, 41.5%, 47.3% and 59.0% at Pb, Cu, Ni, Zn, Pb + Cu, Pb + Ni, Pb + Zn, Cu + Ni, Cu + Zn, Zn + Ni and Pb + Cu + Ni + Zn treatments, respectively.

3.3. Effect of different metal treatments on photosynthetic activities

The photosynthetic efficiency of *S. drummondii* in the presence of different metals was assessed by measuring F_v/F_m and F_v/F_o ratios. The level of response depended on metal types and their combinations. In the present study, F_v/F_m ratios were higher than 0.80 in the Pb, Cu, Zn, Pb + Cu, Pb + Zn, Cu + Ni, Cu + Zn and Ni + Zn

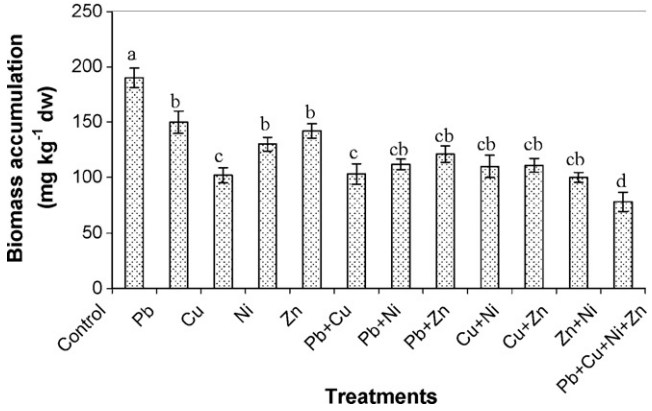

Fig. 1. Biomass accumulation of *S. drummondii* seedlings grown in different metal treatments. Values represent mean ± S.E. ($n = 4$). Different letters on bars indicate significant difference ($P < 0.05$) between means according to Tukey test.

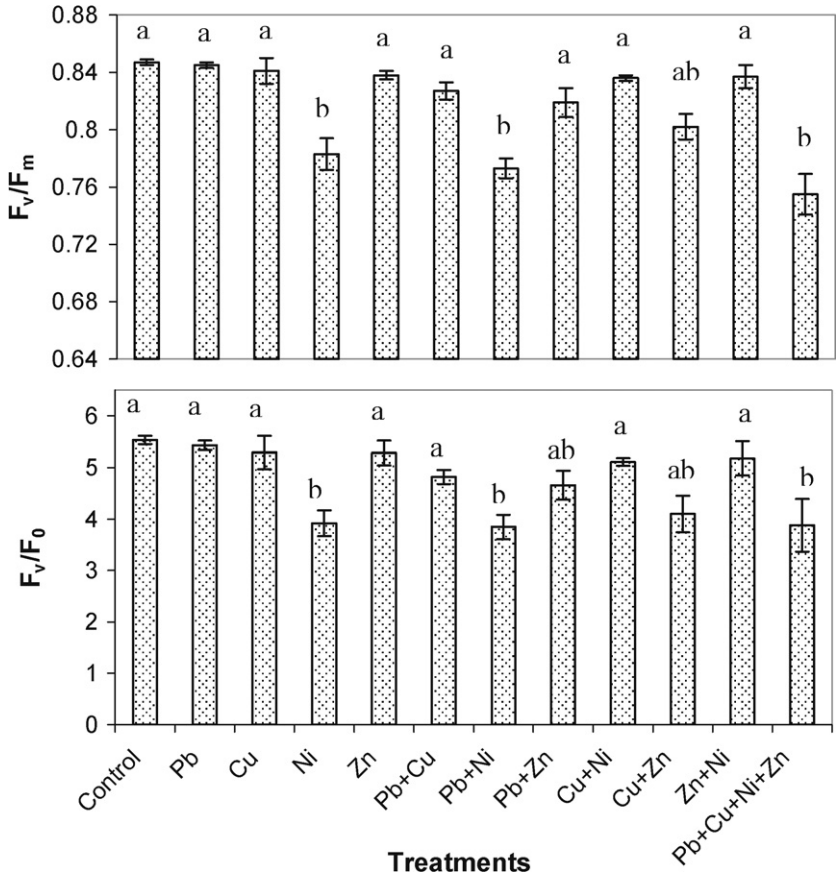

Fig. 2. F_v/F_m and F_v/F_o values of *S. drummondii* seedlings grown in different metal treatments. Values represent mean ± S.E. ($n = 4$). Different letters on bars indicate significant difference ($P < 0.05$) between means according to Tukey test.

treatments (Fig. 2). Though, F_v/F_m ratios were lower than 0.80 in Ni, Pb + Ni and Pb + Cu + Ni + Zn treatments. A similar trend was also exhibited in F_v/F_o values. F_v/F_o ratios were higher than 4.0 in the Pb, Cu, Zn, Pb + Cu, Pb + Zn, Cu + Ni, Cu + Zn and Ni + Zn treatments (Fig. 2). Though, F_v/F_o ratios were lower than 4.0 in Ni, Pb + Ni and Pb + Cu + Ni + Zn treatments.

3.4. Effect of different metal treatments on superoxide dismutase activity

The activity of SOD was significantly ($P < 0.05$) increased in *S. drummondii* seedlings with metal treatments, when compared to the control (no metal) (Fig. 3). However, SOD activities differed with metals as well as with different combinations of metals. Among metals and binary combinations, Cu and Pb + Ni had maximum SOD activity while Pb, Zn and Pb + Zn had the least (Fig. 3). Though, the highest increase in SOD activity was noticed when all four metals were applied together in the mixture (Pb + Cu + Ni + Zn). The SOD activity at Pb + Cu + Ni + Zn treatment was 2.6 folds higher with respect to the control.

3.5. Effect of different metal treatments on ascorbate peroxidase activity

Compared to the control, metal treatments significantly ($P < 0.05$) enhanced the activity of APX in *S. drummondii* seedlings (Fig. 4). However, in general the APX activities were higher in the seedlings treated with a combination of metals as compare to those treated with a single metal. The APX activity showed maximum increase in Pb + Cu + Ni + Zn treatment which was 112% higher than the control. Similar to SOD activity, among metals and binary combinations, Cu and Pb + Ni had highest activation in APX activity, while Zn and Pb + Zn had the least (Fig. 4).

3.6. Effect of different metal treatments on glutathione reductase activity

Fig. 5 shows the influence of different metal treatments on GR activity in *S. drummondii* seedlings. GR activity increased significantly ($P < 0.05$) in all metal treatments but higher activity was noticed when all four metals were applied in combinations. However, no significant ($P < 0.05$) change in GR activity was noticed among the different binary combinations of metal. The activity at

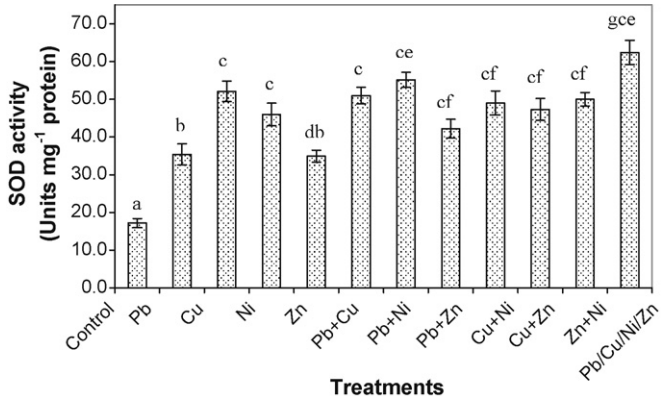

Fig. 3. SOD activity in *S. drummondii* seedlings grown in different metal treatments. Values represent mean ± S.E. ($n = 4$). Different letters on bars indicate significant difference ($P < 0.05$) between means according to Tukey test.

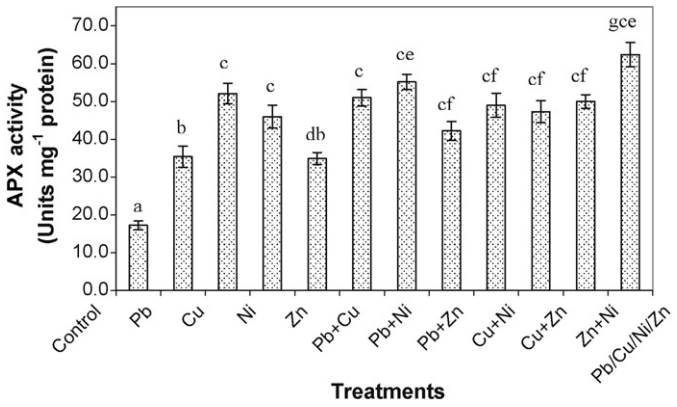

Fig. 4. APX activity in *S. drummondii* seedlings grown in different metal treatments. Values represent mean ± S.E. ($n = 4$). Different letters on bars indicate significant difference ($P < 0.05$) between means according to Tukey test.

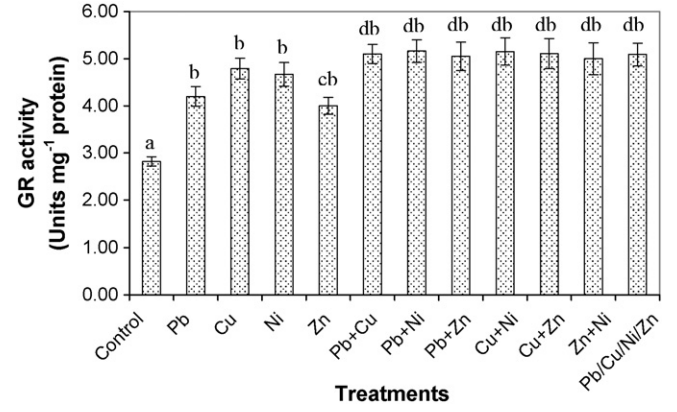

Fig. 5. GR activity in *S. drummondii* seedlings grown in different metal treatments. Values represent mean ± S.E. ($n = 4$). Different letters on bars indicate significant difference ($P < 0.05$) between means according to Tukey test.

Pb + Cu + Ni + Zn treatment increased by 80% with respect to the control (Fig. 5).

3.7. Effect of different metal treatments on glutathione level

Different metal treatments altered the levels of GSH and GSSG in *S. drummondii* seedlings (Table 2). The GSH content as well as GSH/GSSG ratio were found to be higher in all metal treatments when compared with the control (no metal). However, higher levels of GSH and GSSG contents were found in the seedlings treated with binary and quaternary mixture of metals (Pb + Cu, Pb + Ni, Pb + Zn, Cu + Ni, Cu + Zn, Zn + Ni, Pb + Cu + Ni + Zn) as compared to those treated with a single metal. However, the highest increment in GSH content was noticed at Pb + Cu + Ni + Zn treatment. In the Pb + Cu + Ni + Zn treatment, GSH level significantly ($P < 0.05$) increased by 119.7% with respect to the control.

Table 2
Level of reduced (GSH) and oxidized (GSSG) glutathione and GSH/GSSG ratio in the seedlings of *S. drummondii* grown in different metal treatments.

Treatments	GSH (nmol g^{-1}FW)	GSSG (nmol g^{-1}FW)	GSH/GSSG ratio
Control	105.2 ± 4.4^{a}	28.3 ± 1.4^{a}	3.7 ± 0.22^{a}
Pb	180.4 ± 5.2^{b}	40.8 ± 2.0^{b}	4.4 ± 0.20^{b}
Cu	201.0 ± 6.2^{c}	39.1 ± 2.8^{b}	5.1 ± 0.25^{b}
Ni	192.0 ± 6.0^{bc}	38.0 ± 2.4^{b}	5.0 ± 0.30^{b}
Zn	166.4 ± 4.2^{b}	41.0 ± 4.4^{b}	4.0 ± 0.30^{b}
Pb + Cu	210.2 ± 5.0^{c}	44.2 ± 3.8^{b}	4.7 ± 0.24^{b}
Pb + Ni	215.4 ± 5.6^{c}	48.1 ± 4.0^{b}	4.4 ± 0.22^{b}
Pb + Zn	208.0 ± 6.1^{c}	43.4 ± 3.4^{b}	4.8 ± 0.36^{b}
Cu + Ni	222.3 ± 4.5^{cd}	50.7 ± 2.2^{bc}	4.4 ± 0.30^{b}
Cu + Zn	218.7 ± 7.0^{cd}	47.8 ± 3.0^{b}	4.6 ± 0.24^{b}
Zn + Ni	220.1 ± 5.8^{cd}	51.5 ± 5.0^{bc}	4.3 ± 0.32^{b}
Pb + Cu + Ni + Zn	231.2 ± 5.4^{cd}	56.9 ± 4.0^{bc}	4.0 ± 0.28^{b}

Values are the mean of four replicates and within each column, those not followed by the same letter are significantly different ($P < 0.05$).

4. Discussion

There has been a continuing interest in searching for plants that are tolerant to heavy metals and accumulate high amounts of more than one metal. The present study shows that *S. drummondii* plant can accumulate higher concentrations of metals: Pb, Cu, Ni and Zn. Our results also suggest that the uptake of Pb, Cu, Ni and Zn by *S. drummondii* was affected not only by the elements in single applications, but also by the combinations of the elements. The accumulation of Pb was increased in roots as well as in shoots in the presence of Cu, Ni and Zn. Though, Pb showed antagonistic effect on the accumulation of Cu, Ni and Zn in roots, and Cu and Ni in shoots. It may be due to the competition between metals at the plant uptake sites. Earlier reports have shown the increased accumulation of Pb in the presence of Cu and Zn [2,13,14]. However, our results contradict the previous report where total uptake of Pb inhibited in the presence of Cu [28]. Wong et al. [28] reported a reduction in the uptake of Cu in the presence of Pb. In the binary mixture of Cu + Ni and Cu + Zn, copper uptake in the roots was increased in the presence of Ni but significantly ($P < 0.05$) decreased with the presence of Zn, while in the shoots it was reversed. In the presence of Cu, the uptake of Ni and Zn significantly ($P < 0.05$) decreased in the roots, while in the shoots it was increased. These results agree with previous findings in Spring Barley and *Phragmites australis* [29,30]. In a mixture of all metals (Pb + Cu + Ni + Zn), the concentration of Pb, Cu, Ni and Zn in the roots as well as in the shoots significantly ($P < 0.05$) decreased when compared with the individual and binary treatments. It may be due to the competition between metal transport systems during uptake process. It is also possible that co-presence of the metals resulted in a greater reduction in *S. drummondii* growth.

The Pb concentration in *S. drummondii* shoots was well above the threshold level for Pb hyperaccumulator (>1000 mg kg^{-1}) in all the treatments [11]. The concentration of all four metals in *S. drummondii* roots ranged from 42,680 to 64,790 mg of Pb, 9835–15,620 mg of Cu, 481–2104 mg of Ni and 5602–10,360 mg of Zn kg^{-1} tissue dry weight (dw). Though, in shoots the concentrations ranged from 1015 to 1614 mg of Pb, 231–499 mg of Cu, 109–403 mg of Ni and 780–1873 mg of Zn kg^{-1} of dw tissue, respectively. Thus *S. drummondii* may be useful for phytoextraction of Pb [19,20]. In addition, this species may be useful for phytostabilization of Cu, Ni and Zn since roots accumulated high concentrations of these metals.

When a plant is exposed to more than one metal, interactions between metals may occur. Heavy metals may have antagonistic, additive or synergistic effects on plant growth [15,31,32]. In the present study, *S. drummondii* seedlings were grown on the phytotoxic levels of Pb (250 mg L^{-1}), Cu (100 mg L^{-1}), Ni (100 mg L^{-1}) and Zn (300 mg L^{-1}) [22] individually and in combinations. The co-presence of all four metals resulted in an enhanced reduction in *Sesbania*'s biomass compared to the exposure of a single metal, which may suggest a synergistic effect on *S. drummondii* growth. Similar inhibitory effects in co-presence of metals have also been observed in other plant species [13–17,32]. The growth inhibition by metals (Pb, Cu, Ni, Zn) could be due to the high metal accumulation by seedlings (Table 1). In that case, cells might have to spend extra energy to cope with the high metal concentrations in the

tissues [33]. Another reason for reduction in the growth of *S. drummondii* seedlings in the presence of metals may be due to the effect of heavy metals on mineral nutrient uptake and the photosynthesis rate, which was affected by metal treatments in this study. The photosynthetic efficiency of *S. drummondii* in the presence of metals was detected by measuring F_v/F_m and F_v/F_o ratios. F_v/F_m is an indicator of the efficiency of the photosynthetic apparatus, while F_v/F_o indicates the size and the number of active photosynthetic centers in the chloroplast, and therefore, the photosynthetic strength of the plant. An F_v/F_m ratio of 0.8 or above and F_v/F_o ratios of 4.0 or higher indicate that the plant is healthy and not suffering from photosynthetic stress [34]. In other plant species the reduction in photosynthesis has been proposed as being responsible for growth reduction produced by metals [35]. According to Levy et al. [22], shoot metals concentration of 10–100 mg kg^{-1} Pb, 20–100 mg kg^{-1} Cu, 10–100 mg kg^{-1} Ni and 100–400 mg kg^{-1} Zn would be considered toxic to plants. In the present study the metal (Pb, Cu, Ni and Zn) contents in *S. drummondii* shoots greatly exceeded these ranges, indicating that *S. drummondii* may have the ability to tolerate and accumulate high concentrations of Pb, Cu, Ni and Zn without significantly affecting its growth and development.

Heavy metals induce oxidative stress by generating high ROS such as superoxide radical ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) which may result in significant damage to cellular constituents [5]. Many studies have investigated only monometallic responses, and studies on responses against metal interaction are very rare. Plant cells are protected against ROS by the operation of an intricate antioxidant defense system, comprised of both enzymatic [SOD, ascorbate peroxidase (APX), GR, POX] and non-enzymatic anti-oxidants acting as free radical scavenger such as ascorbic acid, cysteine and thiols [36]. The first line of defense against ROS mediated toxicity is achieved by SOD that catalyzes the dismutation of superoxide radicals to H_2O_2 and O_2. In this study, a significant enhancement in SOD activity was observed in the *S. drummondii* seedlings exposed to different metal treatments (Fig. 3), which may be due either to the direct effect of these metals on the SOD related genes or to an indirect effect mediated via an increase in the level of $O_2^{\bullet -}$ radicals [37]. The stimulation of SOD activity has also been reported in several plants subjected to Pb, Cd, Cu, Ni and Zn treatments [37–41]. In plants, peroxidases are essential components of a plant's antioxidant defense system as they break H_2O_2 to water and oxygen. In this study, APX activity was significantly elevated in the metal treated seedlings, which suggest its role in the detoxification of H_2O_2 [42]. However, activity level was higher with metal combinations than individual metal (Fig. 4). Enhanced APX activity has been observed in various plant species after application of Pb, Zn, Cu, Ni and Fe metal [41,42]. However, the inhibition in the peroxidases activity due to heavy metals has also been reported in some plants [43]. Foyer et al. [44] have reported that GR is one of the key enzymes that helps in reduction of GSSG to GSH by oxidizing NADPH to NADP and suggested its crucial role in combating oxidative stress in plant tissues. In the present study we found that GR activity was elevated in *S. drummondii* seedlings exposed to different metal treatments (Fig. 5). The increase in GR activity was also reported with Pb, Cd and Zn treatments in rice and bean [41,45]. Increased activities of SOD, APX and GR in metal treated *S. drummondii* seedlings suggest the tolerance mechanisms developed by this plant.

As antioxidative enzymes, a significant change in the level of GSH was also noticed in *S. drummondii* seedlings exposed to Pb, Cu, Ni and Zn alone and in combinations. As mentioned above, GR catalyzes the NADPH dependent conversion of GSSG to GSH, which is a rate limiting last step of the ascorbate–glutathione pathway. In this study, a considerable change in GR activity supports this hypothesis. A higher GSH/GSSG ratio is necessary to sustain the role of glutathione as an antioxidant and a reductant [5]. Also, it is essential to keep glutathione in its reduced form in order for its incorporation into phytochelatins [46]. In this study, the level of GSH and GSH/GSSG ratios was influenced with single metal and with different metal combination treatments (Table 2). This indicates the potential of *S. drummondii* to tolerate metal inducing oxidative stress. Increased GSH level in metal exposed *S. drummondii* seedlings is in conformity with earlier findings on plants like *Brassica*, *Pteris vittata* L. and *Thlaspi caerulescens* in which enhanced levels of GSH improved Cd, As and Ni tolerance and accumulation, respectively [47–49].

5. Conclusion

This study was conducted to determine the interactive role of Pb, Cu, Ni and Zn on metal uptake, plant growth and anti-oxidative system of *S. drummondii*. Results suggest that the uptake of one metal was affected with the presence of other metals. *S. drummondii* accumulated Pb well above the threshold level for a Pb hyperaccumulator (>1000 mg kg^{-1}), suggesting its usefulness in phytoextraction of Pb. The co-presence of metals resulted in a greater reduction in *S. drummondii* biomass than exposure to a single metal suggesting synergistic or additive response. A coordinated increase in enzymatic antioxidants (SOD, APX and GR) and non-enzymatic antioxidants (GSH) was noticed in response to different metals separately and in combinations. However, compared to the individual metals, induction in enzymatic and non-enzymatic anti-oxidants was higher when metals were applied in combinations, indicating that the co-presence of metals enhanced the oxidative stress. Enhanced level of enzymatic and non-enzymatic anti-oxidants indicates that *S. drummondii* may have a detoxification mechanism to cope with different metals.

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