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Symbiotic role of *Glomus mosseae* in phytoextraction of lead in vetiver grass [*Chrysopogon zizanioides* (L.)]

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ABSTRACT

Lead (Pb) has limited solubility in the soil environment owing to complexation with various soil components. Although total soil Pb concentrations may be high at a given site, the fraction of soluble Pb that plants can extract is very small, which is the major limiting factor for Pb phytoremediation. The symbiotic effect of arbuscular mycorrhizal (AM) fungus, *Glomus mosseae* was examined on growth and phytoextraction of lead (Pb) by vetiver grass [*Chrysopogon zizanioides* (L.)]. A hydroponic study, Phase I (0, 1, 2, and 4 mM Pb) was conducted followed by an incubation pot study, Phase II (0, 400, 800, and 1200 mg kg^{-1} Pb) where vetiver plants were colonized with *G. mosseae*. The results obtained indicate that plants colonized by the AM fungi not only exhibit better growth (increase in plant biomass), but also significantly increase Pb uptake in root and higher translocation to the shoot at all given treatments. Moreover, plants colonized with AM fungi had higher chlorophyll content and reduced levels of low molecular weight thiols, indicating the ability to better tolerate metal-induced stress. Results from this study indicate that vetiver plants in association with AM fungi can be used for improved phytoextraction of Pb from contaminated soil.

1. Introduction

Lead is a highly toxic metal that causes a variety of environmental problems, including loss of vegetation, toxicity in plants and animals and a number of severe health effects in humans, particularly in children under the age of 6 years [1,2]. Primary sources of Pb in soil include industrial and mining activities throughout the world. In addition, elevated levels of Pb in soil result from the use of Pb-based paints, pesticide application, coal burning, gasoline, explosives, anti-spark linings as well as from the disposal of municipal sewage sludge enriched in Pb [3]. The cleanup process for these Pb-contaminated soils represents a significant expense to various industries and governmental agencies [4,5]. Efforts have been made over the last three decades to develop various physicochemical and biological techniques to remediate Pb-contaminated sites [6].

Phytoremediation is a promising technology to remediate soils contaminated with heavy metals and also to facilitate improvement of soil structure [6]. Phytoremediation is a particularly attractive technology since it is relatively inexpensive and environment-friendly compared to traditional engineering practices that rely on intensive soil manipulation [6]. Phytoextraction, removal and concentration of metal into harvestable plant parts have been the major strategy of phytoremediation used to cleanup Pb-contaminated soils [4,5]. Phytoremediation, through continued cultivation and harvesting of selected plant species on Pb-contaminated soils can significantly reduce the soil Pb concentrations. Various strategies to enhance the rate of phytoextraction have been suggested, involving the use of chelating agents to increase the bioavailability of low-solubility metals, genetic engineering and production of transgenic plants with the ability to tolerate and accumulate metals, use of rhizosphere microbes to enhance biomass and metal solubilization, and other agronomic practices [7]. Success of Pb phytoextraction primarily depends on the phytoavailability of Pb, as it must be in either soluble or exchangeable form for plant uptake to occur [8,9]. Lead has limited solubility in the soil environment due to complexation with various organic and inorganic soil colloids, sorption on oxides and clays, and precipitation as carbonates, hydroxides and phosphates [10]. Although total soil Pb concentrations are high in many of the contaminated residential

Abbreviations: AM, arbuscular mycorrhizal; *G. mosseae*, *Glomus mosseae*; LMWT, low molecular weight thiols; ICP-MS, inductively coupled plasma mass spectrometry; MIP, mycorrhizal inoculum percentage; PCR, polymerase chain reaction; bp, base pair.

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sites, the soluble fraction of Pb is often very low, typically less than 1% [1]. Plants have the ability to extract soluble or free forms of Pb rather than the bound ones. Hence, mobilized Pb fraction is a major limiting factor for Pb phytoremediation. Research has focused on artificially inducing Pb desorption from complex soil matrices to enhance Pb phytoextraction. Several synthetic chelating/complexing agents, such as ethylenediaminetetraacetic acid (EDTA), ethylenetrinitrilopentaacetic acid (ETPA), N-hydroxyethylenediaminetriacetic acid (HEDTA), nitrilotriacetate (NTA) and a natural chelant like [S,S^0] ethylenediaminedisuccinate (EDDS), have been used to enhance metal solubility during phytoextraction [1,11,12].

Application of chelating agents in the process of phytoextraction may have potential environmental risks. The major problems include phytotoxic effects of the chelating agents to plants [13], rapid solubilization of metals followed by leaching of the metal–chelate complex to surface and groundwater, toxic effects on soil microorganisms and microfauna, resulting in negative impact on soil ecosystem stability and function [14,15]. Therefore, it is important to develop environment-friendly techniques to enhance the rate of phytoextraction of heavy metals. Keeping these issues in mind, we investigated the Pb tolerance and phytoextraction abilities of vetiver grass (*Chrysopogon zizanioides* L.), a fast growing and high biomass producing plant in the presence of arbuscular mycorrhizal (AM) fungi. Vetiver grass has already been successfully used in our laboratory to phytoextract Pb from Pb-based paint contaminated soils [16,17]. Vetiver grass is a tall (1–2 m), fast-growing, perennial grass, with a long (3–4 m), massive and complex root system, which can penetrate to the deeper layers of the soil [18,19]. Owing to its unique morphological, physiological and ecological characteristics, such as its massive and deep root system and its tolerance to a wide range of adverse climatic and edaphic conditions (including elevated levels of potential toxic elements such as As, Cd, Cu, Cr, Pb, Zn, Se, Zn, Ni, Al, and Mn), interest in this grass has grown over the years [18,20].

Uptake of metal by plants can be influenced by soil microorganisms closely associated with plant roots to form a rhizosphere community [21]. Mycorrhizal fungi are one of the major components of the rhizosphere and form symbiotic associations with most plant species [22]. Of these, AM fungi are the most wide spread mycorrhizae found in both natural and agricultural ecosystems, including heavy metal contaminated sites [23]. Arbuscular mycorrhizal fungi provide a direct link between soil and roots, and consequently may improve nutrition [24] through the formation of extensive extraradical hyphal networks that absorb and translocate nutrients to the roots [25], and modification of the root system that generally results in more extensive and increased branching, leading to increased efficiency in nutrient absorption [26]. In addition, AM fungi have also shown the ability to attenuate biotic and abiotic stresses, including stress produced by heavy metals [27,28]. Furthermore AM fungi also affect metal uptake by plants from soil and translocation from root to shoot; however, the effect depends on various factors such as plant species, fungal species/ecotypes, and soil chemistry [29].

Earlier reports show that Pb is rapidly accumulated in the roots if it is bioavailable in the plant growth media; however, only a small ratio of absorbed Pb is translocated to the shoot [30]. However, for most Pb-contaminated soils, Pb in soil solution is usually less than 0.1% of total soil Pb, limiting Pb availability to the plants [1]. *Glomus mosseae*, an AM fungus, has been reported to have the potential to solubilize minerals, which could enhance metal bioavailability and facilitate root to shoot translocation [31,32]. The objectives of this study were (i) to evaluate the influence of root colonization by the AM fungus *G. mosseae* on Pb uptake by vetiver grass, and (ii) to investigate if interactions between vetiver and *G. mosseae* help the plants better tolerate Pb-induced stress.

2. Materials and methods

2.1. Experimental design

The experiments were performed in two phases—Phase I consisted of plants grown hydroponically and Phase II consisted of plants grown in plastic pots containing soil. The pots were kept in a temperature and humidity controlled incubation chamber during Phase II. An overview of the experimental design is displayed in Table 1. For Phase I experiments, treatments consisted of Pb application at 3 rates in half strength Hoagland solution [33] (0, 1, 2 and 4 mM) in the presence or absence of *G. mosseae*. Each treatment had 3 replicates. The hydroponic set up was kept at room temperature in a 12 h light/12 h dark regimen. Phase II experiments were conducted in an incubation chamber with mean minimum and maximum temperatures of 23 and 28 °C respectively. The experimental design for Phase II consisted of a completely randomized factorial block design; treatments consisted of Pb application at 3 rates (0, 400, 800 and 1200 mg kg^{-1} soil) in the presence or absence of *G. mosseae*. The set up was kept at room temperature in a 12 h light/12 h dark regimen. Each treatment had 3 replicates.

2.1.1. Phase I: hydroponic experiment

ACS reagent grade lead nitrate [$Pb(NO_3)_2$] purchased from Sigma–Aldrich, USA was used as the Pb source. Vetiver plants were purchased from Florida Farms and Nurseries, St. Cloud, FL. The experiment was set up according to Andra et al. [16]. Initially, the vetiver plants were grown in plastic pots, 15 cm deep and 10 cm in diameter containing approximately 150 g potting mixture. After 3 months of acclimation period, the plants were taken out of the soil carefully without damaging the adventitious root system. The plants were thoroughly washed under tap water, wiped dry and growth parameters such as root and shoot lengths and weight were measured. The plants were transferred to containers filled with 1 L each of half strength Hoagland solution consisting of 0.0676 g of KH_2PO_4, 0.253 g of KNO_3, 0.59 g of $Ca(NO_3)_2{\cdot}4H_2O$, and 0.20 g of

Table 1
Experimental design for Phase I (hydroponics study) and Phase II (pot study).

Phase I (hydroponics study)			Phase II (pot study)		
Pb treatments (mM)	AM fungal infection	Replicates	Pb treatments (mg kg^{-1})	AM fungal infection	Replicates
Control (0)	No	3	Control (0)	No	3
1.0	No	3	400	No	3
1.0	Yes	3	400	Yes	3
2.0	No	3	800	No	3
2.0	Yes	3	800	Yes	3
4.0	No	3	1200	No	3
4.0	Yes	3	1200	Yes	3

$MgCl_2 \cdot 6H_2O$ per liter [33]. Compressed air was used to provide the plants with adequate oxygen. Plants were allowed to acclimatize for 2 weeks in Hoagland solution and then inoculum of *G. mosseae* was added as described in Section 2.3. After 2 weeks in contact with the AM fungi, approximately 3 cm long root samples were collected to determine the rate of inoculation. Root and shoot lengths and weights of all the plants were recorded. The plants were transferred to solutions spiked with Pb and grown for 20 d. Root and shoot samples were collected at the beginning of the experiment (time-0), after 10 d (time-mid), and after 20 d (time-final). The plants were harvested, washed and dried and root and shoot lengths and biomass and the rate of infection were recorded. The total Pb concentrations in the root and shoot tissues were determined after acid digestion [35] using a Perkin Elmer Elan-9000 inductively coupled plasma mass spectrometry (ICP-MS).

2.1.2. Phase II: pot experiment

The pot experimental set up was similar to that of the hydroponic experimental set up, except that after the plants were acclimatized in half strength Hoagland solution and inoculated with *G. mosseae*, they were transferred to plastic pots containing soil. The root and shoot lengths were measured, and the plants were weighted prior to the transfer. Before the experiment, the soil was air-dried and passed through a 2 mm sieve and then sterilized at 121 °C at 15 psi (lbf/in^2) for 45 min using Yamato SM 200 sterilizer to ensure complete removal of naturally occurring AM fungi and its spores [31]. Although autoclaving may alter some of the soil properties, it is still the preferred method of sterilization, as it is inexpensive, easy to perform and causes minimal effects on soil physico-chemical properties that might influence Pb sorption and availability [36,37] when compared to other methods. After sterilization, the soil was spiked with $Pb(NO_3)_2$ to achieve concentrations of 400, 800 and 1200 mg kg^{-1} Pb (mass of Pb/mass of dry soil). The plants were allowed to grow for 28 d in the spiked soil. Root and shoot samples were collected at the beginning of the experiment (time-0), after 14 d (time-mid), and after 28 d (time-final). The plants were harvested, washed and dried and root and shoot lengths and biomass and the rate of infection were recorded. The total Pb concentrations in the root and shoot tissues were as described above.

2.2. Soil analysis

The soil used in the Phase II experiment was a Millhopper series Spodosol, collected from the surface horizon of the University of Florida Campus at Gainesville, FL. Soil pH was measured using EPA Method 9050 [35] at soil/water ratio of 1:1. Soil organic matter (SOM%) was determined using the loss-on-ignition method, particle size (clay, sand and silt %) was determined using the pipette method [38], electrical conductivity (EC), Ca, Mg, P, and amorphous oxides (oxalate-extractable) of Fe and Al were determined using standard protocols [38]. Total Pb concentrations in soils were determined by acid digestion following EPA Method 3050B [35]. Plant-available Pb concentrations under acidic conditions were determined by Mehlich-3 extraction (1:8, soil:extractant ratio) method [39]. $NaHCO_3$ extraction using the Olsen method [40] was determined to assess the plant-available Pb and P under alkaline conditions. Exchangeable fraction of Pb was analyzed following Plassard et al. [41]. DTPA-extractable Pb was extracted with 1.9 g diethylenetriaminepentaacetic acid and 14.9 g triethanolamine in 1 L deionized water, pH 7.3 [20]. A Perkin Elmer Elan-9000 model, inductively coupled plasma mass spectrometer (ICP-MS) was used for elemental determinations. Needed QA/QC procedures were followed to assure a recovery of 90–110% of spikes and standards.

2.3. Inoculation of G. mosseae

The *G. mosseae* spores were obtained from University of West Virginia. The isolation of *G. mosseae* was carried out following the standard procedure: a spore dilution of 10^2 was prepared in sterile saline and 1 mL of this suspension was serially diluted up to 10^5–10^7 dilutions. These were plated in nutrient agar medium and incubated at 27 °C for 48 h. After the plants were acclimatized in Hoagland solution, they were exposed to *G. mosseae*. This was done by placing each of the agar plates containing the *G. mosseae* at the bottom of conical containers containing 1 L of half strength Hoagland's solution [33]. The plants were placed directly on top of the agar plates, with the roots touching the nutrient agar media containing the AM fungi. The vetiver plants were allowed to grow for 2 weeks, and were periodically checked for the development of inoculation under a microscope. A separate set of plants were grown for 2 weeks on nutrient agar medium without any *G. mosseae* placed in 1 L of Hoagland's solution to provide a set of controls with general microbial population free of *G. mosseae*.

2.4. Staining procedure and determination of the rate of inoculation

Twenty pieces of root samples (approximately 3 cm long) from each plant were taken to determine the rate of inoculation. The roots were stained by aniline blue dye according to Hebert et al. [42]. The root samples were washed, patted dry and incubated in 10% KOH at 80 °C in water bath for 15 min. The samples were removed from the water bath and a drop of 30% H_2O_2 was added to each sample and incubated for 10 min at room temperature. The roots were rinsed with DI water and transferred to a beaker containing 10 mL of 10% HCl for 5 min. The roots were then transferred to a beaker containing 10 mL of a 0.05% aniline blue solution prepared in 85% lactic acid. The beakers were heated in a water bath for 30 min and then kept in 10 mL of 85% lactic acid solution for 5 min. This procedure stained the fungi blue to allow for microscopic inspection. To prepare slides of root sections, the aniline blue stained roots were carefully sliced with a sterile surgical blade. The thin sections of the roots were placed on slides with sterile DI water and covered with a pre-autoclaved glass slide cover.

The samples were immediately wet-mounted and examined under a compound microscope at a magnification of 100×. A mycorrhizal inoculum percentage (MIP) bioassay was carried out according to Sylvia [43]. The total number of visible cells and the number of infected cells were counted. The percent infection was determined by the following formula:

$$\text{Rate of infection} = \frac{\#\,\text{inoculated cells}}{\text{total}\,\#\,\text{cells}} \times 100$$

On an average, 61% of the roots were found to be colonized by *G. mosseae*. The slides were photographed by a digital microscope camera (Pixera-Penguin 600CLM monochrome) using bright field microscopy at 100× magnification and an oil immersion lens.

2.5. Analytical procedures

2.5.1. Plant digestion

After harvesting, the root and shoot tissues were dried in an oven at 60 °C for 3 d. The dried tissues were digested using nitric acid to determine total Pb content according to Carbonell et al. [34].

2.5.2. Chlorophyll activity

Extraction of chlorophyll from vetiver plants was carried out using 90% acetone in water (v/v). One gram of air-dried plant tissue

was homogenized with 5 ml of acetone. The homogenate was filtered through 0.2 μm filter, and the residue was similarly extracted once again and filtered. The two filtrates were combined and the final volume was made up to 10 mL. All extracts were assayed using Bio-Rad Benchmark Microplate reader. Chlorophyll a and b contents (mg g^{-1} dry weight) were calculated by absorbance values at 663 nm (D663) and 645 nm (D645) using the formula of Arnon [44].

2.5.3. Total thiol and acid-soluble thiol analysis

The flash frozen plant tissues from Phase II experiments were used for estimation of thiols. Total thiols in plant tissues were analyzed according to Hartley-Whitaker et al. [45]. One gram of plant tissue was ground in liquid nitrogen followed by addition of 10 mL of 0.02 M EDTA. The homogenate was centrifuged at 12,000 rpm at 4 °C for 10 min and 0.5 mL of the supernatant was transferred to a test tube and mixed with 1.5 mL of 0.2 M Tris buffer (pH 8.2), 100 μL of 0.01 M DTNB, and 7.9 mL of methanol. The mixture was incubated at room temperature for 10 min before absorbance was measured at 412 nm using a Bio-Rad Benchmark Microplate spectrophotometer. A sample blank (minus DTNB) and a reagent blank (minus sample) were also prepared and measured. Reduced glutathione (Sigma Chemicals, Sigma–Aldrich, St. Louis, MO, USA) in the concentration range of 5–20 μmol was used as standard. Thiol concentrations were calculated by using the extinction coefficient obtained from the standard curve.

2.5.4. Vetiver root DNA extraction and amplification

Vetiver plant tissue flash frozen in liquid nitrogen and stored in −80 °C was used. For DNA extraction, 0.5 g of the sample was ground to a fine powder using liquid nitrogen. The ground samples were transferred to 1.5 mL eppendorf tubes and 700 μL of extraction buffer was added (DNeasy 96 Plant Kit, Qiagen, USA). The tubes were incubated at 40 °C in water bath for 20 min followed by vigorous vortexing for 5 min. The tubes were centrifuged at 8000 rpm for 15 min and the supernatant was discarded. To the pellet, 600 μL of lysis buffer (Qiagen AP1 buffer, Qiagen, USA) was added and resuspended. The tubes were incubated for 30 min at 65 °C in a water bath, and the tubes were periodically inverted to facilitate mixing. The tubes were centrifuged at 12,000 rpm and the supernatant was discarded. The pellet was washed with 500 μL of 70% ethanol and after drying was resuspended in 50 μL TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0).

For each of the isolated DNA sample, PCR was carried out in a 100 μL reaction volume. Each tube contained 2 μL of template DNA, 10 μL dNTP mixture, 10 μL Primer, 20 μL 5× Taq buffer, 0.5 μL Taq polymerase enzyme, and 67.5 μL of DI water. Amplification was carried out using a DNA thermal cycler programmed at 95 °C for 5 min (initial denaturation), 19 cycles at 94 °C for 30 s (denaturation), 56 °C for 30 s (primer annealing), 72 °C for 2 min (extension), and 72 °C for 5 min (final extension). PCR products were separated on 1.5% agarose gel and stained with ethidium bromide, observed under UV light and photographed using a Fotodyne gel documentation system. The following primer pair, designed from *G. mosseae* DNA was used: forward primer 5′-GCAGAAAAAGCCAAGACTCA-3′; reverse primer 5′-TCACATCTTACTATGTCCATATC-3′.

2.5.5. Statistical analysis

Q-tests were performed on all data to eliminate possible outliers at the 95% confidence interval. Mean values ($n = 3$) were determined along with their standard deviations. Appropriate ANOVA analyses were performed using the statistical software JMP IN version 5.1 [46]. A Tukey–Kramer honest significant difference (HSD) test was used to evaluate significant differences among treatment means. Separation of means was conducted individually for each initial Pb concentration.

Table 2
Physico-chemical properties of nutrient solution at different time intervals in the presence of AM fungi, *G. mosseae*. Data are expressed of mean ($n = 3$) ± 1 standard deviation.

Properties	Time-0 (initial)	Time 10 d (mid)	Time 20 d (final)
pH	6.25 ± 0.22	6.60 ± 0.16	6.82 ± 0.15
EC[a] (μs/cm)	286.2 ± 3.5	288.5 ± 4.8	285.4 ± 2.9
P (mM)	79.2 ± 2.5	72.5 ± 1.9	66.2 ± 2.8
Pb (mM)			
1 mM	1.01 ± 0.4 mM	0.78 ± 0.1 mM	0.54 ± 0.02 mM
2 mM	1.98 ± 0.3 mM	1.52 ± 0.3 mM	1.16 ± 0.1 mM
4 mM	4.02 ± 0.5 mM	2.45 ± 0.4 mM	1.93 ± 0.1 mM

[a] EC = electrical conductivity.

3. Results and discussion

3.1. Properties of nutrient solution

The nutrient solution used to grow vetiver grass hydroponically was monitored for pH, EC, total P, and amount of Pb (mM) at different time intervals (initial, mid, and final) during Phase I experiment (Table 2). No significant difference ($p > 0.05$) was observed in pH and EC values at the various time intervals tested. At time-0 the pH was slightly acidic (6.25 ± 0.22) with EC of 286.2 ± 3.5 μs/cm, whereas at the end of the experiment pH reached near neutral (6.82 ± 0.15) with EC of 285.4 ± 2.9 μs/cm. The total P in nutrient solution decreased with time from 79.2 ± 2.5 to 66.2 ± 2.8 mg/L during the experiment, but no significant difference ($p > 0.05$) was observed in p values from time-initial to time-final. Significant reduction ($p < 0.05$) in the amount of Pb present in the nutrient solution was observed with time in the presence of AM fungi, *G. mosseae* (Table 2).

3.2. Soil properties

The physico-chemical properties of the Millhopper soil before spiking with Pb are listed in Table 3. Millhopper is an acid sandy loam with low pH and high extractable P and Fe + Al. There was no significant difference in pH and EC values ($p < 0.05$) for Millhopper soil after treatment with Pb and AM fungi *G. mosseae* (data not shown). Total as well as water-soluble P decreased with increase of Pb concentration, but no significant difference in the values was observed (data not shown). The presence of relatively high concentrations of phosphate and amorphous Fe + Al oxides counteract the pH effects [47]. The observations are similar to those obtained by Wong et al. (treatment of Pb and Zn in the presence of AM

Table 3
Physico-chemical properties of Millhopper soil before Pb treatment.

Properties	Millhopper
pH	6.4
EC[a] (μs/cm)	145
CEC[b] (C mol/kg)	2356
SOM[c] (%)	4.38
P (mg kg^{-1})	
Mehlich 3	134
Total	4875
Ca + Mg (mg kg^{-1})	
Mehlich 3	886
Total	3155
Fe + Al (mg kg^{-1})	
Oxalate	704
Total	4745

[a] Electrical conductivity.
[b] Cation-exchange capacity.
[c] Soil organic matter.

Table 4
DTPA-extractable fraction in soil amended with Pb in the presence and absence of *G. mosseae*.

Pb added (mg kg^{-1})	AMF infection	Pb (mg kg^{-1})
0	Uninfected	52.3
	Infected	60.2
400	Uninfected	349
	Infected	356
800	Uninfected	609
	Infected	615
1200	Uninfected	896
	Infected	905

fungi and vetiver grass) [20] and Chen et al. (treatment of Pb, Zn, and Cd with vetiver grass) [48]. For soils amended with Pb total as well as DTPA-extractable Pb increased with increase in the Pb treatments (Table 4). No significant difference was observed in the DTPA-extractable Pb after the inoculation of AM fungi *G. mosseae*. Wong et al. [20] also showed no effect on DTPA-extractable Pb after the inoculation of AM fungi *G. mosseae* in the soil.

3.3. Rate of infection

The rate of infection was determined for all plant samples used in Phase I and Phase II experiments at the beginning and the end of the experiments. No significant ($p > 0.005$) change was noticed in the rate of infection between the initial stage and the final harvest of the plant samples (Table 5). It was observed that with increase in Pb concentration, the ability of AM fungi *G. mosseae* to colonize vetiver roots increased. Similar results were reported in vetiver grass by Wong et al. [20]. This could be due to the possible P precipitation by added Pb [49]. There are several reports regarding the adverse effect of soil P on mycorrhizal colonization [50,51]. Two possible mechanisms have previously been suggested for the observed decline in mycorrhizal colonization due to increased soil P; decrease in root exudates which in turn affects colonization [52] and direct effect on the development of myccorhizal hyphae [53]. Before the start of the experiments, the presence of AM fungi *G. mosseae* in the roots was verified by performing microscopy (Fig. 1). Fig. 1A shows an uninfected vetiver root section, whereas Fig. 1B shows infected root of vetiver grass with *G. mosseae* stained with aniline blue.

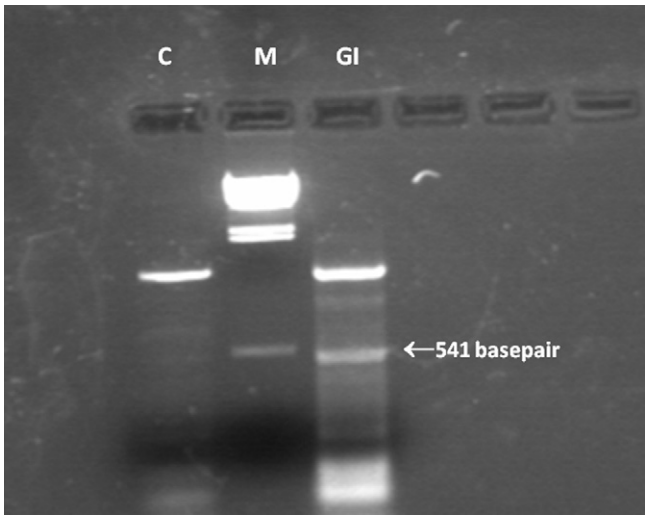

Fig. 2. Ethidium bromide stained agarose gel showing the products of PCR reactions performed using DNA extracted from *G. mosseae* infected vetiver root. Primers were designed from a Glomus-specific DNA sequence. A 541 bp sequence was amplified from DNA isolated from infected roots, confirming fungal colonization in vetiver root. Lane C is control vetiver root without infection. Lane M—Hind III digested λ DNA marker. Lane GI shows the vetiver root infected with *G. mosseae* with the 541 bp long *G. mosseae* DNA.

3.4. Confirmation of G. mosseae inoculation by PCR

Several reports have shown that spores and/or sporocarps of AM fungi *G. mosseae* in plant tissue can be confirmed using aniline blue

Table 5
Mean rate of infection as a percentage for inoculation of AM fungi, *Glomus mosseae* at time-0, time-mid and time-final for Phase I and Phase II experiments. Data are expressed of mean ($n = 3$) ± 1 standard deviation.

Phase I (hydroponics study)				Phase II (pot study)			
Pb treatments (mM)	Time-0	Time-mid	Time-final	Pb treatments (mg kg^{-1})	Time-0	Time-mid	Time-final
Control (0)	0.00	0.00	0.00	Control (0)	0.00	0.00	0.00
1.0	0.00	0.00	0.00	400	0.00	0.00	0.00
1.0	66.00 ± 3.0	64.50 ± 2.5	62.50 ± 3.5	400	63.50 ± 2.5	62.00 ± 2.0	59.50 ± 2.5
2.0	0.00	0.00	0.00	800	0.00	0.00	0.00
2.0	68.00 ± 3.5	68.50 ± 3.0	63.00 ± 2.5	800	66.00 ± 3.5	62.00 ± 2.5	58.50 ± 2.5
4.0	0.00	0.00	0.00	1200	0.00	0.00	0.00
4.0	66.50 ± 2.5	65.00 ± 3.0	61.50 ± 2.5	1200	64.00 ± 2.0	62.00 ± 2.0	59.50 ± 2.5

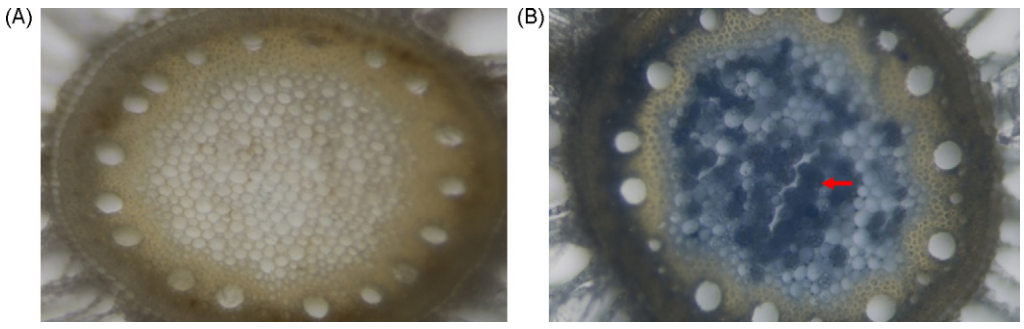

Fig. 1. Microscopic images (100× magnification) showing the presence and absence respectively of AM fungi, *Glomus mosseae* in uninfected (A) and infected (B) vetiver root. Root sections were stained using aniline blue. Arrow indicates fungal hyphae in vascular bundles.

Table 6
Root and shoot dry biomass of vetiver grass in the presence and absence of AM fungi, *G. mosseae* for time-mid and time-final for Phase I and Phase II. Data are expressed of mean ($n = 3$) ± 1 standard deviation.

Pb treatment	AMF *G. mosseae*	Time-mid		Time-final	
		Root wt. (g)	Shoot wt. (g)	Root wt. (g)	Shoot wt. (g)
1 mM	Uninfected	25.72 ± 0.5	15.28 ± 0.4	25.87 ± 0.3	14.13 ± 0.2
	Infected	27.24 ± 0.5	16.76 ± 0.4	27.49 ± 0.4	18.51 ± 0.3
2 mM	Uninfected	25.45 ± 0.2	13.55 ± 0.3	25.57 ± 0.2	12.43 ± 0.4
	Infected	26.64 ± 0.4	15.36 ± 0.5	26.84 ± 0.4	18.76 ± 0.5
4 mM	Uninfected	24.4 ± 0.6	13.6 ± 0.7	24.58 ± 0.4	12.42 ± 0.6
	Infected	25.52 ± 0.4	16.48 ± 0.7	25.74 ± 0.4	19.06 ± 0.7
400 mg kg^{-1}	Uninfected	25.8 ± 0.8	15.2 ± 0.9	25.98 ± 0.7	15.02 ± 0.9
	Infected	27.45 ± 0.6	17.55 ± 0.6	27.68 ± 0.4	19.52 ± 0.6
800 mg kg^{-1}	Uninfected	25.43 ± 0.5	12.57 ± 0.4	25.66 ± 0.7	11.34 ± 0.8
	Infected	27.25 ± 0.8	15.75 ± 0.5	27.5 ± 0.6	18.5 ± 0.6
1200 mg kg^{-1}	Uninfected	24.65 ± 0.6	12.35 ± 0.3	24.79 ± 0.4	11.71 ± 0.5
	Infected	26.94 ± 0.5	15.06 ± 0.6	27.19 ± 0.4	17.61 ± 0.6

staining, diamidinophenylindole (DAPI) staining, and florescence microscopy [42]. However, Giovannetti et al. [54] reported that due to improper or poor growth of AM fungi examinations can be difficult and erroneous. In the present study, we conducted PCR using DNA isolated from infected and uninfected vetiver roots to further confirm the inoculation of *G. mosseae* (Fig. 2). The samples were subjected to PCR using primers designed from a Glomus-specific DNA sequence, which amplified the expected 541 bp sequence from root DNA of infected plants (Fig. 2) but not uninfected plants.

3.5. Plant growth and G. mosseae colonization

Colonization of AM fungi, *G. mosseae* had a significant and positive impact on the growth of vetiver plants at all treatments (Table 6). The observations are consistent with those of previous workers [20,57]. The presence of *G. mosseae* increased both root and shoot biomass significantly ($p < 0.005$) at all tested Pb concentrations in both Phase I and Phase II experiments (Table 6). In Phase I experiments, the highest plant growth compared to uninfected plants was observed in the presence of *G. mosseae* at 1 mM Pb concentration (15%) followed by a decline at 2 mM (14%) and 4 mM (12%) Pb concentrations (Fig. 3A). Similar results were observed in Phase II experiments; plants grown in soil spiked with 400 mg kg^{-1} Pb had highest plant growth with *G. mosseae* colonization (18%) followed by 800 mg kg^{-1} (15%) and 1200 mg kg^{-1} (13%) Pb concentration (Fig. 3B). In spite of the downward trend with increasing Pb concentration, no significant difference ($p < 0.005$) was observed in mean plant growth response between the Pb treatments with *G. mosseae* colonization. On the other hand, in uninfected plants (without *G. mosseae* colonization) decrease in the plant growth was observed as a result of Pb treatment. While no decline was observed in Phase I plants treated with 1 mM Pb when compared to 0 mM Pb, a decline of 5% and 7.5% was observed in plants treated with 2 and 4 mM Pb respectively (Fig. 3A). Similar trends were observed in Phase II experiments, except for 400 mg kg^{-1} Pb concentration. While a slight increase in biomass was observed at 400 mg kg^{-1} Pb (2.5%), there was a decline in plant biomass at 800 mg kg^{-1} Pb (7.5%) and 1200 mg kg^{-1} Pb (8.75%) (Fig. 3B).

The results obtained above are in agreement with some of the previous studies, which have also reported increase in plant biomass due to the presence of AM fungi [31,52]. Agely et al. [31] colonized Chinese brake fern (*Pteris vittata* L.) with AM fungi, *G. mosseae* for phytoextraction of arsenic (As) (0, 50, and 100 mg kg^{-1}) from soil. They demonstrated that use of AM fungi, *G. mosseae* increased the plant biomass with increase in frond's mass by nearly 100% compared to uninfected plants at highest

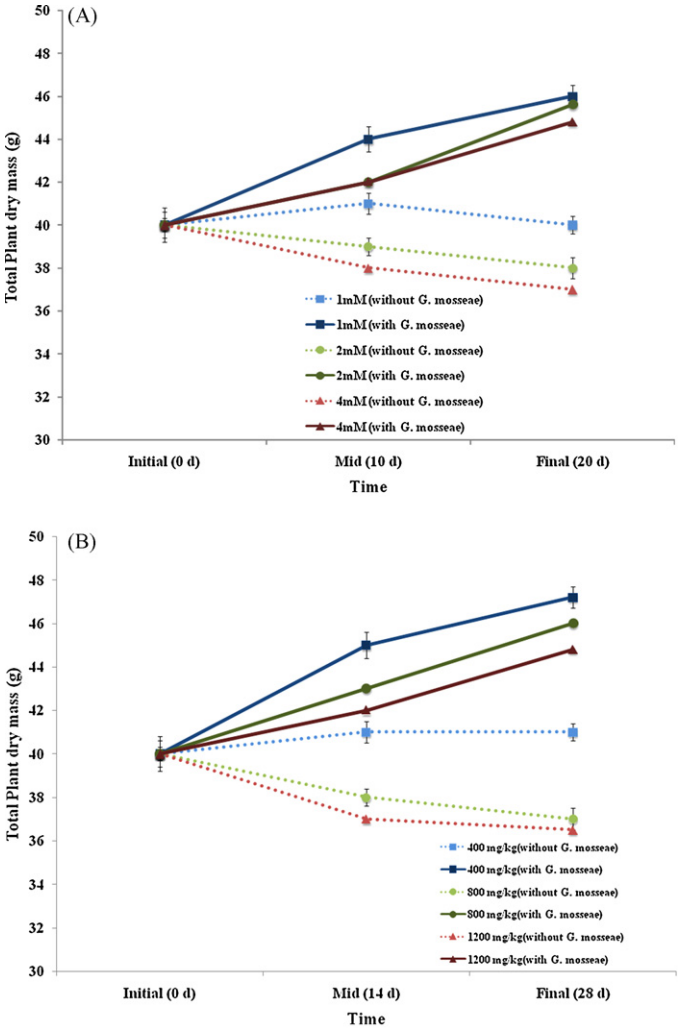

Fig. 3. (A) Comparison of total plant biomass (root and shoot) in infected (*with G. mosseae*) and uninfected (*without G. mosseae*) vetiver plants for Phase I at various Pb concentrations (1, 2 and 4 mM). Data points are expressed as means of three replicates ± one standard deviation. (B) Comparison of total plant biomass (root and shoot) in infected (*with G. mosseae*) and uninfected (*without G. mosseae*) vetiver plants for Phase II at various Pb concentrations (400, 800, and 1200 mg kg^{-1}). Data points are expressed as means of three replicates ± one standard deviation.

(100 mg kg^{-1}) As treatment. They also demonstrated significant increase in plant biomass at all given As treatment. Chen et al. [57] used four plant species (two native plant species, *Coreopsis drummondii* and *P. vittata*, a turf grass, *Lolium perenne* and a leguminous plant, *Trifolium repens*) in copper mine tailing. They showed that mycorrhizal colonization increased both shoot and root biomass significantly for all the tested plant species, except *L. perenne*. Plants with high biomass are vital for successful process of phytoremediation [6]. The fact that *G. mosseae* colonization significantly increased biomass of vetiver plants in spite of the presence of considerable amounts of Pb indicates the positive influence of plant–microbe interaction on plant growth.

3.6. Effect of G. mosseae on the uptake of lead by vetiver grass

One of the issues that have been encountered in the successful use of phytoextraction of Pb by vetiver grass is that Pb accumulates mainly in root tissue of vetiver [58,59]. Antiochia et al. [58] used vetiver grass for remediation of soil contaminated with Cr, Cu, Pb, and Zn and reported a very high accumulation of Pb in the root tissues, demonstrating lack of translocation. Wilde et al. [59] used vetiver grass for phytoextraction of Pb from firing range soil using fertilizer and chelating agent (EDTA) to mobilize Pb. While these amendments were able to increase the amount of Pb uptake, translocation from root to shoot was still limited.

One of the parameters influencing the uptake of Pb by vetiver grass was determined to be the establishment of *G. mosseae*. Symbiotic interactions with AM fungi have been recognized to benefit plants under environmental stress including heavy metal pollution [60]. In Phase I experiments, there were significant differences ($p < 0.005$) observed in Pb accumulation in the roots of vetiver grass grown in the presence of *G. mosseae* 10 and 20 d from exposure to Pb (Fig. 4A). The highest increase in Pb uptake was observed at 4 mM Pb concentration (98%) followed by 2 mM (87%) and 1 mM (85%) Pb concentrations compared to the uninfected plants (Fig. 4A). As expected, the initial Pb concentrations had a significant effect ($p < 0.005$) on the overall uptake of Pb by the roots.

Results also indicate a significant difference in the overall root to shoot translocation of Pb in vetiver grass grown in the presence of *G. mosseae* (Fig. 4A). An increase in uptake of Pb and rate of translocation was observed with increasing Pb concentration in plants colonized with *G. mosseae*. Highest amount of Pb in shoot was observed in plants treated with 2 mM Pb (70%) followed by 1 mM (65%) and 4 mM Pb (37%) compared to the uninfected plants (Fig. 4A). Similar studies involving *Cannabis sativa* showed an increase in metal translocation with the inoculation of *G. mosseae* [61].

In Phase II experiments, Pb uptake in roots and translocation from root to shoot in plants colonized with *G. mosseae* showed a similar trend as in Phase I. Significant differences in uptake of Pb in roots of plants colonized with *G. mosseae* were observed in all treatments when compared to plants without *G. mosseae*. The highest amount of Pb uptake in plants inoculated with *G. mosseae* was at the 1200 mg kg^{-1} treatment (5500 mg kg^{-1} dry weight) followed by 800 and 400 mg kg^{-1} Pb treatments (3433 and 2278 mg kg^{-1} dry weight respectively) (Fig. 4B). In addition, with an increase in the concentration of Pb the plants were exposed to, translocation of Pb to the shoot also increased in plants inoculated with *G. mosseae* which was significantly higher compared to plants without *G. mosseae* (Fig. 4B). The highest amount of Pb in shoot tissue was seen at 1200 mg kg^{-1} treatment (2179 mg kg^{-1} dry weight) followed by 800 and 400 mg kg^{-1} treatments (1525 and 1070 mg kg^{-1} dry weight respectively). In comparison, in uninfected plants, the 1200 mg kg^{-1} treatment had the highest uptake (1561 mg kg^{-1} dry weight) followed by 800 and 400 mg kg^{-1} treatments (938 and 701 mg kg^{-1} dry weight respectively).

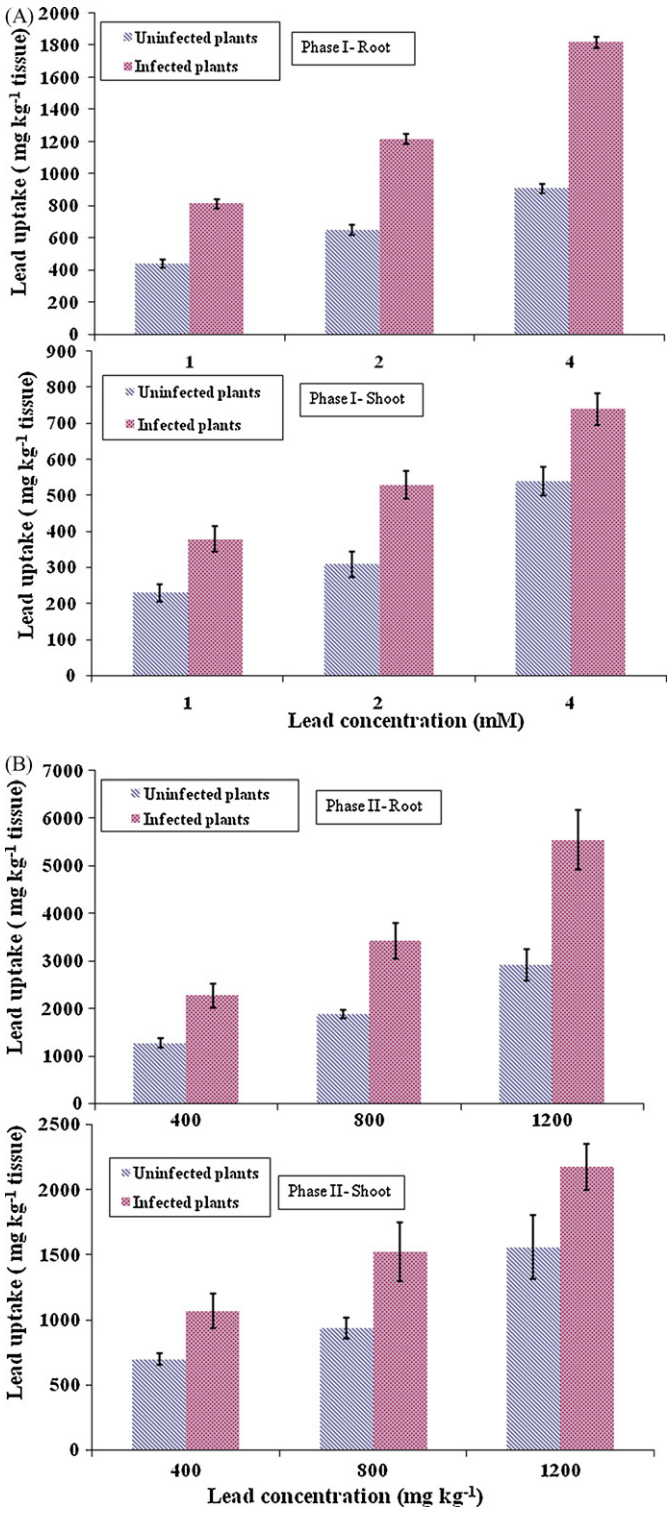

Fig. 4. (A) Comparison of uptake of Pb in vetiver grass in the hydroponic study (Phase I). The plants in the hydroponic study were exposed to 1, 2 and 4 mM Pb and the amount of Pb in root and shoot (A) tissues in infected (*with G. mosseae*) and uninfected plants (*without G. mosseae*) were estimated. Data points are expressed as means of three replicates ± one standard deviation. (B) Comparison of uptake of Pb in vetiver grass in the pot study (Phase II). The plants in the pot study were exposed to soil spiked with 400, 800 and 1200 mg kg^{-1} Pb and the amount of Pb in the root and shoot tissues in infected (*with G. mosseae*) and uninfected plants (*without G. mosseae*) was estimated. Data points are expressed as means of three replicates ± one standard deviation.

In terms of total amount of Pb taken up by vetiver plants, it was found that plants in Phase II contained higher percentages of Pb compared to Phase I. The highest amount of Pb present in Phase I plants was in infected plants treated with 1 mM Pb (12%) which declined to 6.17% in infected plants treated with 4 mM Pb (data not shown). In Phase II experiments, the highest amount of Pb was taken up by infected plants treated with 400 mg kg^{-1} Pb (17.7%) which declined marginally to 15.2% in infected plants treated with 1200 mg kg^{-1} Pb. The amount of Pb contained in infected plants was twice that of uninfected plants in both Phase I and Phase II (data not shown).

These results indicate the positive impact of *G. mosseae* in enhancing not only Pb uptake in vetiver plants but also root to shoot translocation of Pb. It also indicates that Pb concentration in shoot of vetiver can be modulated by AM fungi when growing in soil contaminated with Pb. Earlier studies reported by Agely et al. [31] and Trotta et al. [32] in soil systems showed similar uptake results in plants with inoculation of mycorrhizal fungi. However, the results are different from that observed by Wong et al. [20], as they reported no significant effect of colonization by *G. mosseae* on the uptake of Pb or Zn in vetiver grass at higher concentrations (1000 mg kg^{-1}). The observed difference between the present study and Wong et al. [20] could possibly be due to extrametrical development of *G. mosseae* on vetiver root and/or the physico-chemical properties of the soil used in the experiment.

3.7. Total thiols and acid-soluble thiols, and chlorophyll activity

Like various other heavy metals, Pb exposure influences the behaviors of a wide range of physiological, biochemical and ultrastructural functions in plants. Inactivation of Pb, as well as of other heavy metals, is connected with a detoxification mechanism, which consists of the synthesis of thiol peptides–phytochelatins in plants. Low molecular weight thiols have also been reported to play a crucial role in both metal chelation and detoxification, as well as function as antioxidants to alleviate oxidative stress caused by exposure to metals [62]. In Phase II of the study, we carried out analysis of LMWT and chlorophyll activity to estimate the amount of Pb stress produced in the plants with and without *G. mosseae*. The result indicates that at all concentrations (tested both in root and shoot tissues) there were differences in the amounts of LMWT produced in plants with and without the inoculation of *G. mosseae* (Fig. 5). The amount of thiols produced in plants with inoculation of *G. mosseae* was lower than plants without *G. mosseae*. Significant differences ($p < 0.05$) were seen, especially at higher Pb concentrations (800 and 1200 mg kg^{-1}) both in root and shoot tissues. The lowest amount of LMWT was produced at 1200 mg kg^{-1} Pb treatment (66%) in roots of plants inoculated with *G. mosseae*, followed by 800 and 400 mg kg^{-1} Pb treatments (78% and 80% respectively) compared to uninfected plants (Fig. 5). In shoot tissue of *G. mosseae* infected plants, the lowest amount of LMWT was produced at 400 mg kg^{-1} treatment (64%) followed by 1200 and 800 mg kg^{-1} Pb treatments (65% and 69% respectively) compared to uninfected plants (Fig. 5). This indicates that the presence of *G. mosseae* helped to reduce the amount of Pb-induced stress in vetiver. The effect of AM fungi has been studied in recent years [63,64], on stress caused by heavy metals, such as Cu, Zn, and Cd and it is generally accepted that AM fungi protect the host plant against metal toxicity [58].

Heavy metals, particularly Pb and Cu, have been widely reported to reduce chlorophyll content and photosynthetic efficiency in plants [61–64]. In this study, chlorophyll a and b activities were estimated in vetiver plants in the presence and absence of AM fungi *G. mosseae*. Results show that there were substantial differences in chlorophyll activities in plants with and without inoculation of *G. mosseae* at all concentrations tested (Fig. 6). Plants inoculated with *G. mosseae* had higher chlorophyll a and chlorophyll b

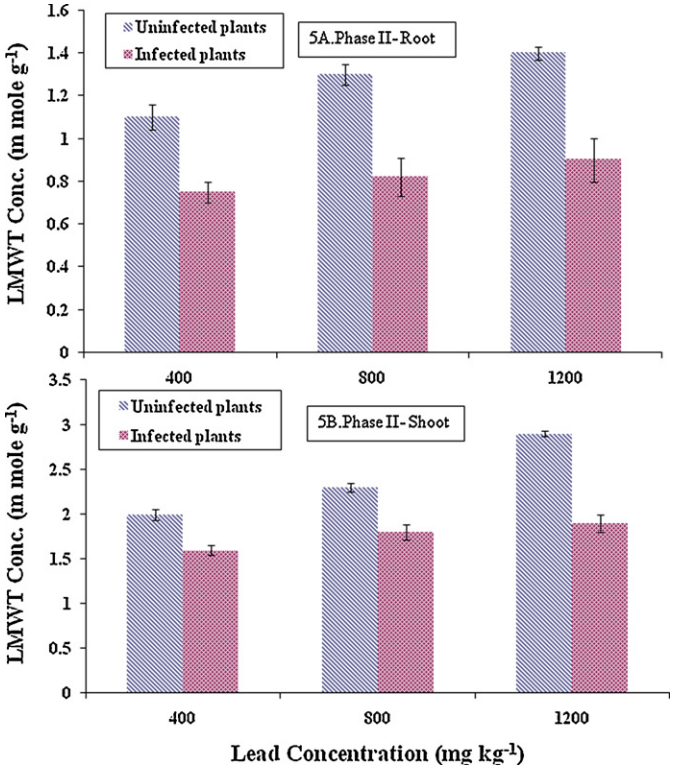

Fig. 5. Comparison of low molecular weight thiols produced in vetiver root (A) and shoot (B) in infected (*with G. mosseae*) and uninfected (*without G. mosseae*) plants during the Phase II study. Data points are expressed as means of three replicates ± one standard deviation.

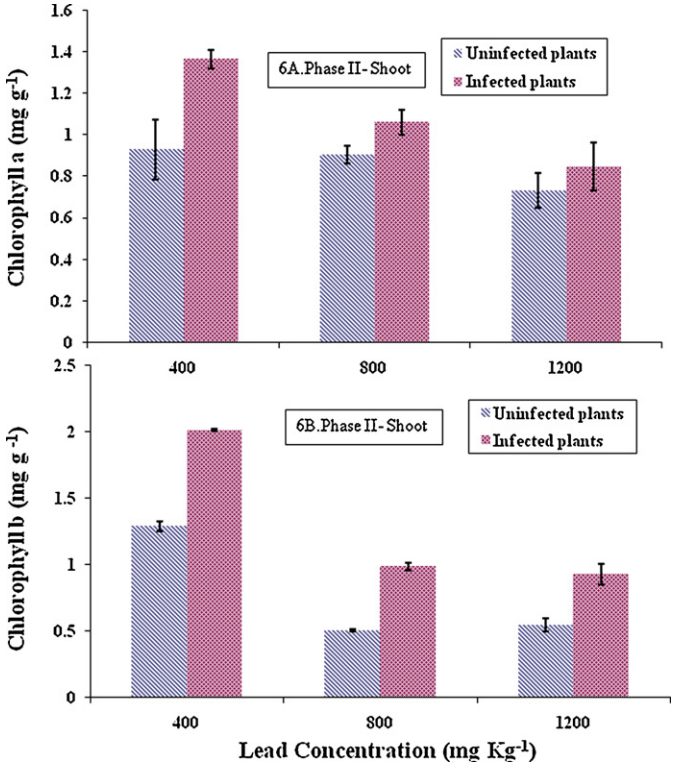

Fig. 6. Comparison of chlorophyll a (A) and chlorophyll b (B) activities in vetiver shoot in infected (*with G. mosseae*) and uninfected (*without G. mosseae*) plants during the Phase II study. Data points are expressed as means of three replicates ± one standard deviation.

activities compared to plants without *G. mosseae*. However, no significant differences ($p > 0.005$) were observed in chlorophyll a and chlorophyll b (Fig. 6) in plants with and without inoculation of *G. mosseae*. "Dilution effect" has been proposed as a possible mechanism by which AM fungi reduce the metal-induced stress in host plants [65]. The protection to the plant may be largely mediated by metal sequestration by AM fungi *G. mosseae*. Cheng et al. [66] have demonstrated that AM fungi, *G. mosseae* improve plant growth by facilitating mineral nutrition in adverse conditions.

4. Conclusions

This study elucidates the potential of beneficial AM fungi, *G. mosseae* in solubilizing Pb, thereby increasing the bioavailability of Pb for uptake by vetiver grass. The use of AM fungi obviates the use of harmful chelating agents which has the potential to contaminate the surrounding environment. Moreover, the results from the analysis of LMWT and chlorophyll activity show that the presence of a community of AM fungi in vetiver roots helped reduce Pb-induced stress thus improving plant growth and Pb uptake. Interesting differences were observed in Pb accumulation in plants grown in hydroponic medium when compared to those grown in soil. In all cases, approximately twice the amount of Pb was taken up in infected plants compared to the uninfected plants. Highest percentage of Pb was taken up in plants grown in soil infected with AM fungi, whereas plants grown hydroponically accumulated comparatively lower levels of Pb. Moreover, *G. mosseae* inoculation resulted in better root to shoot translocation of Pb, which is a major criterion for successful phytoremediation.

The current study was a short-term greenhouse study that indicated the beneficial role of AM fungi in enhancing plant growth, Pb uptake by root and root to shoot Pb translocation. However, longer-term verification of the results is necessary. The mechanisms responsible for increased Pb uptake and translocation in vetiver are still unclear. Moreover, uptake of Pb is strongly influenced by soil physico-chemical properties. Further studies are required to test the efficacy of AM fungi in enhancing metal uptake in soils with varying physico-chemical properties. Further studies are also required to understand the modulation of host gene expression by AM fungi, and its influence on the phytoremediation potential of vetiver grass.

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