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Synergistic effects of arbuscular mycorrhizal fungi and phosphate rock on heavy metal uptake and accumulation by an arsenic hyperaccumulator

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ABSTRACT

The effects of arbuscular mycorrhizal (AM) fungi and phosphate rock on the phytorextraction efficiency of a hyperaccumulator (*Pteris vittata*) and a non-hyperaccumulator (*Cynodon dactylon*) plant were studied. Both seedlings were planted in As contaminated soil under different treatments {(1) control (contaminated soil only), (2) indigenous mycorrhizas (IM), (3) mixed AM inoculum [indigenous mycorrhiza + *Glomus mosseae* (IM/Gm)] and (4) IM/Gm + phosphate rock (P rock)} with varying intensities (40%, 70% and 100%) of water moisture content (WMC). Significant As reduction in soil (23.8% of soil As reduction), increase in plant biomass (17.8 g/pot) and As accumulation (2054 mg/kg DW) were observed for *P. vittata* treated with IM/Gm + PR at 100% WMC level. The overall results indicated that the synergistic effect of mycorrhiza and P rock affected As subcellular distribution of the hyperaccumulator and thereby altered its As removal efficiency under well-watered conditions.

1. Introduction

Long-term mining activities have produced large areas of tailings, causing pollution and land degradation. Amongst all mining methods, flotation has been the most widespread method of metal ore enrichment [1], involving both mechanical and chemical processing of metalliferous minerals. However, such a method may give rise to unfavourable air–water conditions for plant growth by restricting water infiltration during rainfall and decreasing water recharge by capillary rise from deeper layers during dry periods [2]. Thus, variation of water content in soil is considered to be one of the most important abiotic factors limiting plant growth and yield in the field [3]. Consequently, most metals in contaminated soil are less available to plants, e.g., in waterlogged soils, the solubility of some metalloids such as As is generally reduced due to the low redox potential [4] and formation of sparingly soluble sulfides [5]. Such phenomenon may also affect the phytoextraction efficiency of metal hyperaccumulators in the field.

Arsenic is a crystalline metalloid that exists in several forms and states. Its toxicity and mobility in the environment is dependent on both its chemical form and species [6]. Total As concentration alone is insufficient to assess its environmental impact in contaminated soils. Henceforth, measuring available form of As in soil is a better indicator to reflect the phytotoxicity of As [7]. Sequential extraction [8] has been commonly used to assess both As availability and its mobility in soils. However, for soil As, due to its chemical similarity to P, the method used for P fractionation has also been used specifically for As fractionation [9]. Soil As is operationally separated into four fractions: water-soluble plus exchangeable As (We–As, using NH_4Cl), Al-bound As (Al–As, using NH_4F), Fe-bound As (Fe–As, using NaOH) and Ca-bound As (Ca–As, using sulphuric acid) because As retention in soil is related to the content of extractable amorphous and crytostalline hydrous oxides of Fe and Al and exchangeable Ca in soil [10]. Based on the sequential extraction, information about the retention and partitioning of As in soils can be estimated. Even though sequential extraction suffers from a lack of specificity during chemical fractionation and the resorption of dissolved metals by soils during the extraction, it is still a useful tool to evaluate metal bioavailability in soils [9].

Mine wastes usually contain high levels of toxic metals and low levels of nutrients unsuitable for plant growth. It has been indicated that AM fungi can colonize plant roots in metal contaminated soil [11–23] and it has been commonly observed that AM fungi increases shoot uptake of metals [24–25] in severely contaminated soils. In addition, AM fungi could protect plants against harmful effects of metals [26] and respond to water deficit at morphological, anatomical and cellular levels with modifications. This will allow the plant to avoid stress or to increase tolerance [27] so as to alleviate the stress symptoms. There are a number of principle strategies used by mycorrhizal plants to deal with toxic metal cations in contaminated soil: (1) Compartmentalization strategies: As is translocated to subcellular compartments (invariably the vac-

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uole) where they can be stored in places such as the vacuole and the cell wall away from the cytoplasm [28,29]. In addition, under the influence of AM fungi, more compounds (metal chelators such as amino acids, phytochelatins and metallothioneins) present in the cell may be stimulated for transporting metals within the plant [30]; (2) Avoidance strategies: plants and mycorrhizas may inhibit certain metals entering their cells and thereby reduce the host exposure to toxic metals [30]. Therefore, understanding the subcellular distribution of As is essential in elucidating the mechanisms involved in metal tolerance and metal hyperaccumulation of these plants. It will help to explain the effects of soil moisture regime on cellular activities as well as define the possible roles of mycorrhiza in aiding its host with regards to As accumulation in adverse environments. Mycorrhizas colonized in the plant can be effective in ameliorating metal toxicity on the host plant [31] by changing the distribution of As in the cells of the plant. The mechanisms employed by the higher plant at the cellular level to accumulate heavy metals may probably be similar to some of the strategies employed by fungi, namely binding to extracellular materials or sequestration in the vacuole compartment [32]. It was also claimed that indigenous AM fungi are able to protect host plants under adverse environmental conditions by enhancing water exchange between soil and plant [33,34]. Furthermore, arbuscular found in AM fungi can increase water uptake and assist plants to tolerate water stress conditions [35] and also increase the adhesion capacity of the soil aggregates, thereby contributing towards soil stability for plant growth [2]. Therefore, mycorrhizal hyperaccumulating plants are able to adapt to such adverse soil environments by altering redox potential and releasing metal chelating compounds into the rhizosphere, and efficiently absorbing them into the root and translocating them to the shoot [36,37]. In addition, mycorrhizal symbiosis may also assist host plants in using essential elements that are unavailable to non-mycorrhizal plants and therefore contributing to plant growth under water deficit conditions [38]. However, the fungal strains isolated from highly contaminated sites may grow slowly, with a long lag phase, lower efficiency in utilizing nutrients, and with diversed nutritional requirements or altered pH of the growth media (potentially changing metal ion speciation and toxicity) [30]. It was revealed that both fungal isolates and plants may be varied in their metal accumulation ability under different soil conditions. Thus, the use of AM fungi to enhance plant growth in metal contaminated soils may require careful selection of specific fungal isolates. The skillful use of a suitable amendment to maximize plant growth and to capitalize on interactions or competitions between metals and elements such as P is needed in order to increase the adaptability of plants in field sites.

There has been a growing interest in the role of mycorrhizas in affecting biological regulation of plant production and phytoextraction efficiency. Recently, the role of mycorrhizal fungi in soil C and N dynamics has been extensively studied [39–41]. However, the role of mycorrhizas in passive processes, such as water moisture content in contaminated soil is less well understood. The objectives of the experiment were therefore to (1) determine whether mycorrhizal inoculum and phosphate rock amended to As contaminated soil could enhance plant accumulation of As under different soil moisture contents and (2) assess the strategies of mycorrhizal hyperaccumulator plant for As accumulation compared with a non-hyperaccumulator plant, when grown in As contaminated soil.

2. Materials and methods

2.1. Plant selection

Metal hyperaccumulators *Pteris vittata* (Chinese brake fern) from Dashunlong (DSL) mining site, *Equisetum sp* (Horsetail) from Chongyang mining site and *Sedum alfredii* from a Hangzhou mining site were sampled. Three replicates of each plant species together with its rhizosphere soil were collected. The soil and whole plant, including roots and shoots, were washed with double distilled water, freeze-dried and ashed (at −40 °C, 10 days). All samples were used to analyze total As [digested by concentrated nitric acid and hydrochloric acid at 5:1 (v/v), and measured by Inductively Coupled Optical Plasma-Emission Spectrometry (ICP-OES) (Optima 3000DV, Perkin-Elmer, USA)] [42]. Biological Absorption Coefficient (BAC = shoot/soil concentration) [43] and Accumulation Factor (AF = shoot/root concentration ratio) [44] of plants were subsequently determined in order to choose the best hyperaccumulator for further experiment.

2.2. Suitability of Glomus mosseae as a mixed AM inoculum

The main objective of this experiment was to test whether *G. mosseae* can survive in As contaminated soil. Sieved, air-dried contaminated soil (200 g) was placed in each pot (8 cm × 8 cm) under different proportions [clean soil:contaminated soil = 100:0, 25:75, 50:50, 75:25, 0:100 (w/w)]. The method for preparing mycorrhizas was based on information provided by the supplier [Biorize (Dijon, France)]. *G. mosseae* inoculum (10 g) was inoculated in each pot with 1 kg of contaminated soil and mixed well by burying at 5 or 10 cm below the surface soil. Ten *Zea mays* seeds obtained from the Hong Kong Kadoorie Farm were germinated in a greenhouse at a temperature of 18–25 °C and relative humidity of 60–80%. *Z. mays* was used to serve as trap culture to produce sufficient amounts of mycorrhizal spores and promote faster sporulation compared with the other two plants (i.e. *P. vittata* and *C. dactylon*) investigated based on our observations. There were a total of 25 pots consisting of a serial mixture of polluted soil and clean soil (polluted soil:clean soil = 100:0, 75:25, 50:50, 25:75, 0:100; w/w) each with five replicates, arranged under a randomized block design in a greenhouse. After 90 days, fresh plant root was harvested, cut and mixed using a forcep. Mycorrhizal infection in plant root was determined under a light microscopy (C011, Olympus, United Kingdom) [45]. For further verification of *G. mosseae* colonized in plant root, Polymerase Chain Reaction (PCR) was employed to screen the target mycorrhizal gene. Root sample was immersed with liquid nitrogen and extracted by using DNeasy plant mini kit (Qiagen Hilden, Germany). The purity of the extracted DNA was checked at 260 and 280 nm, and DNA products were stored at −20 °C. PCR primers for *G. mosseae* were designed with some modifications [46]. The sequences of NDL22 and GM521 were 5′TGGTCCGTGTTTCAAGACG3′ and 5′CCTTTTGAGCTCGGTCTCGTG3′, respectively. PCR conditions were 5 min at 94 °C, 35 cycles of 20 s for 94 °C, 30 s for 58 °C, and 30 s for 72 °C, followed by 5 min for 72 °C by PCR machine (GeneAmp 9700, Applied Biosystem, USA). PCR products were electrophoresed on 2% agroase gel with 1× TBE buffer (8.9 mM Tris-borate, 0.2 mM EDTA) by using Horizontal DNA Electrophoresis Gel Boxes (Bio-Rad, USA), and analyzed under an ultra-violet light after staining with ethidium bromide, using Standard Gel Documentation System (EDU 170–17347, Bio-Rad, USA).

2.3. Effects of soil moisture on the uptake of As by P. vittata and C. dactylon

Contaminated soil (a sandy loam with light reddish brown color) was collected from the surface soil (0–10 cm) at DSL mining site. The soil was air-dried and sieved through a mesh screen (2 mm) to remove rocks and other large organic materials. pH (soil:distilled water = 1:2) was determined using a Beckman pH meter and total organic matter was determined by loss-on-ignition method [47]. Total As in soil was determined after extraction with concentrated 50% HF/HCl/HNO_3 [48] using a microwave accelerated reaction sys-

Table 1
Experimental design for pot trial ($n = 4$).

Soil moisture levels (% of WMC)	Amount of mycorrhizal inoculum/phosphate rock used (g)			
	Control	IM[a]	IM/Gm[b]	IM/Gm + PR[c]
40	N/A	10	10	10/10
70	N/A	10	10	10/10
100	N/A	10	10	10/10

[a] IM = indigenous mycorrhizas in DSL mine site.
[b] IM/Gm = mixed AM inoculum (indigenous mycorrhizas + *G. mosseae*).
[c] IM/Gm + PR = mixed AM inoculum + phosphate rock amendment.

tem (Mars-5, CEM Corporation, USA). Soil samples were stored at room temperature (24 °C) for further experiment.

To determine the soil water holding capacity (WHC), triplicate soils were placed into a soil sieve with a nylon mesh net (0.2 mm). The soil samples were saturated with distilled water and allowed to drain for 18 h while the top was sealed with a plastic wrap to prevent evaporation. At 18 h, the soil in the sieve was weighed and recorded. Soils were then dried overnight at 80 °C for 24 h followed by drying at 104 °C for 2 h and reweighed. The water moisture content (WMC) of soil prior to drying was defined at 100% field capacity.

The experimental set up is listed in Table 1. Sieved soil (400 g) was used for each pot. In order to adopt the appropriate range of WMC used in the experiment, a preliminary test was conducted by planting *P. vittata* and *C. dactylon* seedlings into soil which had a range of WMC of 40%, 70% and 100%. A WHC ranging from 0% to 30% was too low to support growth of both plants. All pots were gravimetrically adjusted with autoclaved distilled water once every 2 days to maintain WMC before the experiment. There were four replicates for each treatment.

After establishment of the soil moisture treatments, seedlings of *P. vittata* and *C. dactylon* (sampled from DSL As mining site) (pregerminated for 3 months) were transferred to the pots. Water was added to each pot every day to maintain WMC throughout the duration of the study. All the pots were placed in a greenhouse (average temperature of 24 °C and 40–60% relative humidity) under a randomized block design for a period of 2 months.

2.3.1. Analytical procedures

2.3.1.1. Soil. Soil samples were used to analyze total As using the same procedures described above. Arsenic speciation of soil was conducted by sequential extraction [49]. Soil (2 g) and extraction solution (40 ml) were used in each step. Concentrations for the fractions of WE–As, Al–As, Fe–As, and Ca–As were obtained by analyzing the supernatants extracted using 1 M NH_4Cl (shaken for 0.5 h), 0.5 M NH_4F (0.5 h), 0.1 M NaOH (17 h), and 0.5 M H_2SO_4 (17 h), respectively. Between each fraction, 25 ml of saturated sodium chloride was added to the solid residue, re-suspended, centrifuged at 1381 × *g* by bench-top centrifuge (Allegera 6R, Beckman, USA), and the supernatant discarded. For each step, the suspensions, after having been shaken for a specified time, were centrifuged at 1381 × *g*. Arsenic concentration in all fractions were measured by ICP-OES (Optima 3000DV, Perkin-Elmer, USA).

2.3.1.2. Subcellular distribution of As in plant. Plants were washed and separated into above ground and below ground parts. Part of the plant sample was used to study subcellular As distribution while the other part was used to determine the total As concentration. Subcellular distributions of As were determined according to the methods described by Hans and Pathore et al. [50,51]. Samples were homogenized in pre-cooled homogenized solution (0.25 mmol l^{-1} sucrose, 50 mmol l^{-1} Tris-maleate buffer) and 1 mmol l^{-1} $MgCl_2$. The final pH of the solution was adjusted to 7.8 using 1 M sodium hydroxide (NaOH). All homogenization and subsequent fractionation were performed on ice. The mixture was washed three times with homogenized solution. The homogenate was topped up to a volume of 20 ml homogenized solution and transferred into a 50 ml centrifugal tube, then centrifuged at 500 × *g* for 2 min using a high speed refrigerated centrifuge (J2, Beckman, USA). The pellet obtained was considered as the cell wall fraction. The supernatant was further centrifuged at 20,000 × *g* for 45 min to separate the cell organelles; thus the pellet from this second centrifugation was considered as the cell organelle fraction. The resultant supernatant solution was considered as the cytoplasmic supernatant fraction which included macromolecular organic matter and inorganic ions in the cytoplasm and vacuoles [52]. All samples of each fraction and the rest of the plant tissues were freezed-dried and digested using the method described above. The As concentration was measured using ICP-OES. Appropriate blanks and standard tissue samples were run for quality control. Standard reference materials for plant (Tomato leaves, NIST-1573a) and soil (Montana II Soil, NIST-2711) purchased from the Natural Institute of Standard and Technology (NIST) were used for the QA/QC program and the recovery rate of As ranged from 90% to 110%.

2.3.2. Morphological features of plant root

Fine lateral roots were cut into 1 cm pieces, heated for 20 min at 100 °C in 10% potassium hydroxide to remove cytoplasm and then stained using lactophenol blue [45]. The stained roots were then mounted on glass slides (10 pieces of root per slide) for examination under a compound microscope (C011, Olympus, United Kingdom) (100–400×) which was mounted with an eyepiece cross-hair that could be moved to randomly select positions. Colonization percentage of mycorrhizae was estimated for each sample by examining 1 cm long pieces of roots. Arbuscular mycorrhizal structures within the colonized root length were recorded and arbuscular richness was calculated [53].

2.4. Statistical analyses

The data were subjected to one-way ANOVA using SPSS 11.0 software. Means and standard derivations were calculated based on four replicates. Means were compared by Duncan's Multiple Range Test and T-test at 0.05 significance level [54].

3. Results

3.1. As uptake by plants sampled at mine sites

In general, all plant species examined contained high concentrations of As (239–14,408 mg/kg DW) in accordance with the high metal concentrations found in the field soils (Table 2). In addition, most of the shoot samples exhibited higher ($P < 0.05$) As concentrations (482–14,408 mg/kg DW) than the corresponding root samples (239–1592 mg/kg DW). *P. vittata* had the highest biological absorption coefficient (BAC) of 0.871 ($P < 0.05$) among the three plant species (*P. vittata*, *Equisetum sp* and *S. alfredii*) indicating As was

Table 2
Arsenic accumulation in hyperaccumulators ($n = 3$).

Plant species	Common name	Sampling location	As in plants (mg/kg) DW		As in soil (mg/kg) DW	Biological absorption coefficient[a]	Translocation factor[b]
			Frond/shoot	Root			
Pteris vittata	Chinese brake fern	Dashunlong	14408 ± 104	1592 ± 57.5	16540 ± 101	0.871	9.05
Equisetum sp.	Horsetail	Chongyang	4422 ± 15.8	3649 ± 29.7	4809 ± 106	0.759	1.21
Sedum alfredii	–	Hangzhou	482 ± 171	239 ± 127	1403 ± 16.5	0.170	2.02

[a] Calculated by (shoot/soil concentration ratio).
[b] Calculated by (shoot/root concentration ratio).

Table 3
Results of mycorrhizal colonization for *G. mosseae* in *Zea mays* grown in different proportion of contaminated soil based on microscopy. Means with the same letter are not significantly different according to Duncan Multiple Range test at 5% level.

Percentage of contaminated soil (%)	0	25	50	75	100
Mycorrhizal infection (%)	39.7 ± 8.24a	29.0 ± 1.03ab	27.7 ± 0.11ab	14.7 ± 1.86b	4.80 ± 2.38c

effectively taken up when compared with the other two hyperaccumulators.

The As Translocation Factors (TFs) were generally larger than 1, indicating that As was retained in the aboveground parts of plants. The values of TFs were: *P. vittata* (9.05) > *S. alfredii* (2.02) > *Equisetum sp* (1.21).

3.2. Detection of G. mosseae colonized in plant roots

According to Fig. 1, a band was found in all proportions of contaminated soil in which a pair of PCR primers were designed to be specific for *G. mosseae*. These primers, designed for PCR application, had a melting temperature of 58 °C and amplicon size of 360 bp. The specific primers amplified the 360 bp fragments from their respective target templates, but not those from the non-target template. Interestingly, the band density for PCR also showed a linear response to mycorrhizal infection in root (Table 3). The results indicated that this fungal strain was able to survive and colonize plant root in all levels of As contaminated soil. Henceforth, *G. mosseae* was used for preparing mixed AM inoculum.

3.3. As removal and soil As reduction by plants

3.3.1. Soil properties

The contaminated soil collected from the DSL mining site had a pH of 7.46, total organic carbon of 2.5% with low electrical conductivity (92 μS/cm), low levels of total and water-soluble N and P, but elevated As concentration of 9623 mg/kg DW (Table 4).

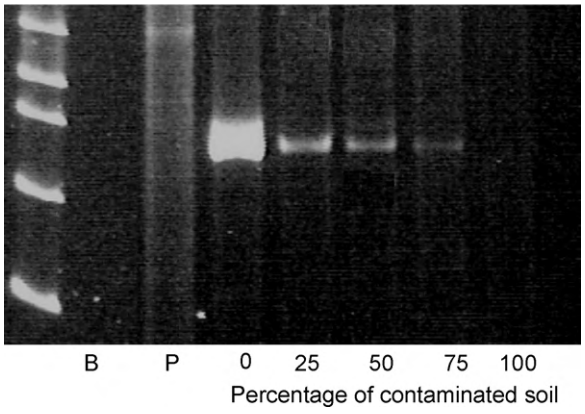

Fig. 1. PCR amplification of *G. mosseae* performed by NDL22 and GM521 primer pair. From left to right: Lane 1: DNA marker; Lane 2 (B): control; Lane 3 (P): plant grown in 0% contaminated soil without mycorrhizal inoculation; Lane 4 (0): mycorrhizal plant grown in 0% of contaminated soil; Lane 5 (25): mycorrhizal plant grown in 25% of contaminated soil; Lane 6 (50): mycorrhizal plant grown in 50% of contaminated soil; Lane7 (75): mycorrhizal plant grown in 75% of contaminated soil; Lane 8 (100): mycorrhizal plant grown in 100% of contaminated soil.

After 8-week plantation, lower concentrations of As in all amended soils were noted when compared with the non-amended soil (Fig. 2). A significant decrease of As concentration in soil was observed (23.8% of soil As reduction) when planted with mycorrhizal *P. vittata* in all As-contaminated soil at 100% WMC. All As levels in soil detected were significantly ($P < 0.05$) decreased in IM/Gm + PR treatment regardless of the difference in the original soil moisture. However, no significant changes ($P < 0.05$) of As concentration in soil was found with *C. dactylon* for all treatments (Fig. 2).

In general, the range of As reduction in soil was 1.95–23.8% by *P. vittata* and 0.34–4.54% by *C. dactylon*, respectively (Table 5). The percentage of As reduction from soil for *P. vittata* was dramatically increased in each level of WMC in soil by comparing with control in IM/Gm + PR treatment. However, no such phenomenon was found in *C. dactylon*.

3.4. Fractionation of soil As

Among the four soluble-forms of As in soil, sulfuric acid (Ca–As), sodium hydroxide (Fe–As), and ammonium fluoride (Al–As) extracted much more As than ammonium chloride (We–As) from As contaminated soil (i.e. Ca–As > Fe–As > Al–As > We–As). Ca–As was the dominant species present in the soil regardless of the levels of WMC and all treatments (control, IM, IM/Gm and IM/Gm + PR) used in soil. For soil planted with *P. vittata*, Ca–As showed the greatest reduction and at the same time the proportion of Fe–As and Al–As fractions increased when treated with IM/Gm + PR for soil at 100% WMC (Fig. 3). Interestingly, As concentrations in Fe–As and Al–As fractions was generally lower in amended soil than in non-amended soil after *C. dacylon* was planted.

Table 4
Physiochemical characteristics of soil used for experiment (Mean ± SD; $n = 4$).

Soil properties	Results
Soil pH	7.46 ± 0.01
Soil EC (μS/cm)	92 ± 8
OM (%)	2.50 ± 0.32
Total N (mg/kg)	18.2 ± 1.11
Water-extractable N (mg/kg)	0.11 ± 0.05
Total P (mg/kg)	5.82 ± 0.81
Water-extractable P (mg/kg)	0.10 ± 0.01
Total K (mg/kg)	875 ± 19
Water-extractable K (mg/kg)	1.52 ± 0.52
Total As (mg/kg)	9623 ± 112

Table 5
Soil arsenic reduction (%) in different treatments after growing *P. vittata* and *C. dactylon* for 8 weeks in an arsenic-contaminated soil (from DSL mining site).

% of WMC in soil	*P. vittata*				*C. dactylon*			
	Control	IM	IM/Gm	IM/Gm + PR	Control	IM	IM/Gm	IM/Gm + PR
40	1.95 ± 0.77c	5.99 ± 6.47c	7.99 ± 5.56b	11.4 ± 1.38a	1.71 ± 1.47a	0.79 ± 0.93a	0.42 ± 0.497a	1.50 ± 0.476a
70	2.50 ± 1.18b	15.3 ± 1.41a	16.2 ± 1.59a	14.9 ± 1.25a	3.83 ± 0.50a	0.34 ± 1.26a	1.10 ± 2.21a	1.69 ± 0.582a
100	2.95 ± 1.13c	17.3 ± 1.55b	18.5 ± 1.96b	23.8 ± 1.70a	4.54 ± 2.64a	2.14 ± 1.21a	1.39 ± 2.08a	1.51 ± 1.28a

Values are expressed as mean ± standard derivation with $n = 4$.
Within the same row of each plant species at the same percentage of WMC, means with the same letter are not significantly different according to Duncan's Multiple Range test at 5% level.
IM = indigenous mycorrhizas in DSL mine site.
IM/Gm = mixed AM inoculum (indigenous mycorrhizas + *G. mosseae*).
IM/Gm + PR = mixed AM inoculum + phosphate rock amendment.

3.5. Plant biomass

In general, *P. vittata* had a higher biomass than *C. dactylon* at all levels of WMC and all treatments (control, IM, IM/Gm, IM/Gm + PR). By comparing with different treatments, IM/Gm + PR was able to improve the plant biomass of both plants significantly ($P < 0.05$) for soil at all WMC levels (Table 6).

3.6. Plant uptake

After 8 weeks, As mainly accumulated in the shoots rather than in the roots for *P. vittata*, but it was the opposite for *C. dactylon* (Fig. 4). The amounts of As accumulated in *P. vittata* generally increased in the order of 40% WMC < 70% WMC < 100% WMC. *P. vittata* accumulated significantly ($P < 0.05$) higher concentrations of As in frond when treated with IM/Gm + PR for soil at 100% WMC (2054 mg/kg DW). However, no significant ($P > 0.05$) differences were found for As concentrations in roots for all treatments.

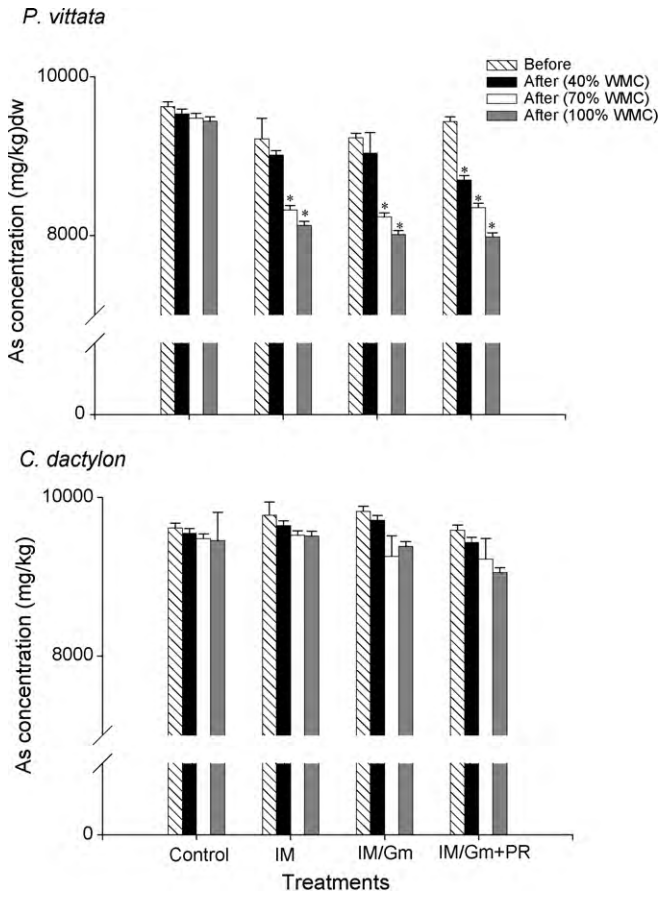

Fig. 2. Arsenic concentration in soil after growing *P. vittata* and *C. dactylon* for 8 weeks. Within the same treatment, mean followed by * indicates results are significantly different at 5% level according to t-test.
IM = indigenous mycorrhizas in DSL mine site.
IM/Gm = mixed AM inoculum (indigenous mycorrhizas + *G. mosseae*).
IM/Gm + PR = mixed AM inoculum + phosphate rock amendment.

3.7. As distribution in subcellular fractions of plants

Figs. 5 and 6 show the percentages of As subcellular distribution in different parts of the plant after the experiment. Regardless of WMC, As distribution in plant followed the descending order of cytoplasmic matrix > cell wall > organelle, except high As concentration was found in the cell wall of the root tissue in *C. dactylon* (Fig. 6). *P. vittata* in IM/Gm + PR treatment had more As distributed in the cytoplasmic matrix fraction than the other compartments in frond tissue when it was planted in the soil at 100% WMC. This indicated that the cytoplasmic matrix in frond tissue exhibited a preferential affinity for As than the other compartments in *P. vittata*.

3.8. Mycorrhizal colonization and aruscular richness

Table 7 shows the mycorrhizal infection rates and arbuscular richness in plant roots. The infection percentage ranged 1.71–23.8%, 1.81–34.0%, 4.17–43.5% in *P. vittata* and 1.81–21.5%, 6.01–35.1%, 5.21–56.7% in *C. dactylon* for the soils at levels of 40%, 70% and 100% WMC, respectively. A significant increase in colonization was observed for plants grown in treated soil (control, Im/Gm, IM/Gm +

Table 6
Effects of mycorrhizal infection on the biomass (g/pot) of *P. vittata* and *C. dactylon* ($n = 4$).

% of WMC in soil	Control	IM	IM/Gm	IM/Gm + PR
P. vittata				
40	6.17 ± 1.48c	7.14 ± 0.23c	8.74 ± 0.67b	12.5 ± 0.78a
70	7.53 ± 0.31c	8.53 ± 2.31bc	11.7 ± 0.46b	16.3 ± 0.59a
100	6.54 ± 0.82d	8.21 ± 0.68c	13.0 ± 1.75b	17.8 ± 0.52a
C. dactylon				
40	2.73 ± 0.48c	4.37 ± 0.80b	7.64 ± 0.52ab	8.43 ± 0.67a
70	3.09 ± 1.18c	6.42 ± 0.67b	7.59 ± 0.21a	8.83 ± 0.81a
100	4.07 ± 0.71b	7.11 ± 1.42ab	8.85 ± 2.36ab	10.4 ± 0.52a

Values are expressed as mean ± standard derivation with $n = 4$.
Within the same row of each plant species at the same percentage of WMC, means with the same letter are not significantly different according to Duncan's Multiple Range test at 5% level.
IM = indigenous mycorrhizas in DSL mine site.
IM/Gm = mixed AM inoculum (Indigenous mycorrhizas + *G. mosseae*).
IM/Gm + PR = mixed AM inoculum + phosphate rock amendment.

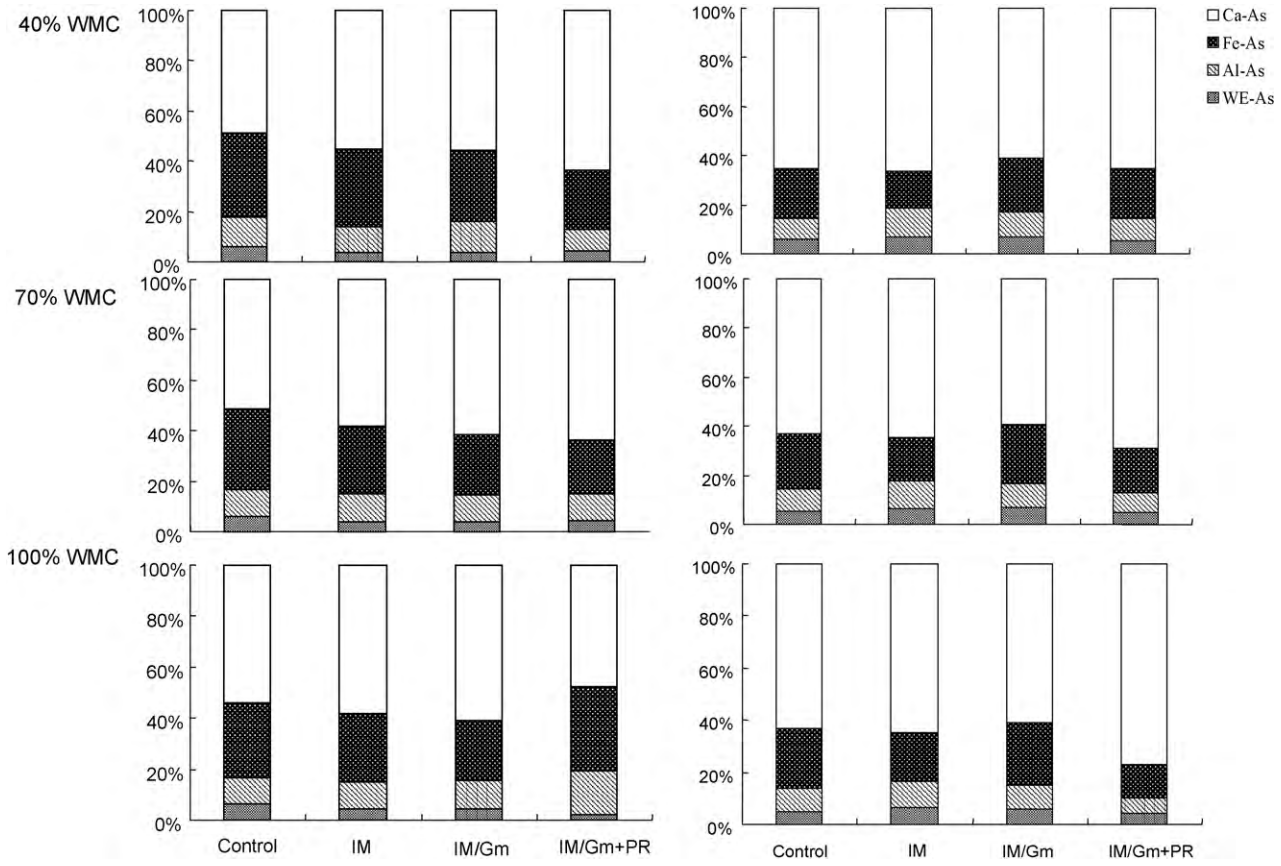

Fig. 3. Arsenic fractionation after growing *P. vittata* and *C. dactylon* 8 weeks under different treatments in different WMC levels of arsenic contaminated field soil. WE–, Al–, Fe–, and Ca–As stand for associated with water-soluble plus exchangeable Al, Fe, and Ca respectively.
IM = indigenous mycorrhizas in DSL mine site.
IM/Gm = mixed AM inoculum (indigenous mycorrhizas + *G. mosseae*).
IM/Gm + PR = mixed AM inoculum + phosphate rock amendment.

Table 7
Percentages of mycorrhizal colonization and arbuscular richness of *P. vittata* and *C. dactylon* planted on As contaminated soil collected from DSL ($n = 4$).

% of WMC in soil	Control	IM	IM/Gm	IM/Gm + PR
P. vittata				
Total mycorrhizal colonization (%)				
40	1.71 ± 5.09b	12.5 ± 4.63ab	23.8 ± 7.30a	22.7 ± 7.02a
70	1.81 ± 0.98b	17.1 ± 4.36ab	22.2 ± 6.69a	34.0 ± 8.59a
100	4.17 ± 2.80c	25.4 ± 1.88b	34.0 ± 8.59b	43.5 ± 2.85a
Arbuscular richness				
40	N.D.	2.58 ± 0.94	3.83 ± 1.70	7.90 ± 1.04
70	N.D.	3.36 ± 1.17	4.24 ± 3.16	8.78 ± 1.09
100	N.D.	N.D.	2.29 ± 1.13	13.9 ± 5.04
C. dactylon				
Total mycorrhizal colonization (%)				
40	1.81 ± 9.40c	13.1 ± 9.54b	22.1 ± 1.15a	21.5 ± 1.17a
70	6.01 ± 0.68c	26.8 ± 11.0b	32.2 ± 1.86a	35.1 ± 1.04a
100	5.21 ± 0.79c	27.5 ± 2.10b	47.1 ± 7.93a	56.7 ± 2.39a
Arbuscular richness				
40	N.D.	1.88 ± 0.17	2.63 ± 1.29	5.75 ± 0.59
70	N.D.	3.13 ± 1.54	3.11 ± 1.74	7.86 ± 0.79
100	N.D.	5.34 ± 1.58	6.61 ± 1.80	9.77 ± 0.58

N.D. = non-detectable.
Values are expressed as mean ± standard derivation with $n = 4$.
Within the same row of the same WMC for each plant species, means with the same letter are not significantly different according to Duncan's Multiple Range test at 5% level.
IM = indigenous mycorrhizas in DSL mine site.
IM/Gm = mixed AM inoculum (indigenous mycorrhizas + *G. mosseae*).
IM/Gm + PR = mixed AM inoculum + phosphate rock amendment.

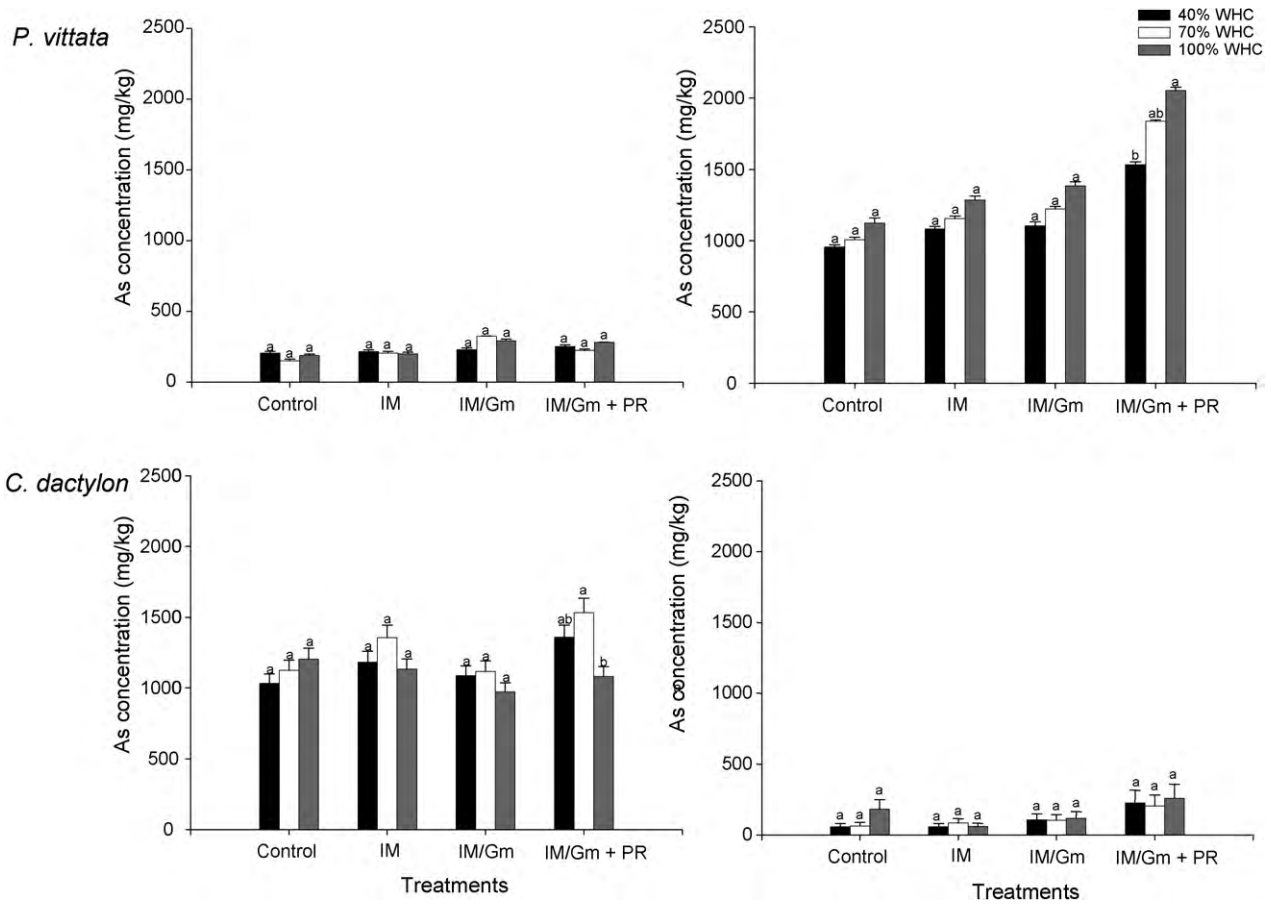

Fig. 4. Arsenic distribution in root (left) and shoot (right) of *P. vittata* and *C. dactylon* at different WHC of arsenic contaminated soil. Within the same treatment, means with the same letter are not significantly different according to Duncan's Multiple Range test at 5% level.
IM = indigenous mycorrhizas in DSL mine site.
IM/Gm = mixed AM inoculum (indigenous mycorrhizas + *G. mosseae*).IM/Gm + PR = mixed AM inoculum + phosphate rock amendment.

PR) than control soil at all levels of WMC. For arbuscular richness, no arbsucular formation was found for both plants in control treatment irrespective of WMC levels of soil. The arbuscular richness was generally higher in *P. vittata* (40% WMC: 7.90; 70% WMC: 8.78; 100% WMC: 13.9) than in *C. dactylon* (40% WMC: 5.75; 70% WMC: 7.86; 100% WMC: 9.77) in IM/Gm + PR treatment. The results indicated that the percentage increase in root infection was generally parallel to the amount of arbuscular formed within the plant roots.

4. Discussion

4.1. Potential of using mycorrhizas to aid its host for phytoremediation

The present results showed that mixed AM inoculum could improve As translocation in the contaminated soil in comparison with the control. In addition, the successive symbiosis between mixed AM inoculum and plant (shown in this experiment) presented positive effects on tissue hydration and plant physiology [33] and also increased the absorbing area of mycorrhizal root for water uptake [55]. Therefore, there seems to be amble evidence confirming the contribution of IM/Gm symbiosis for the maintenance of water balance for plants surviving in As contaminated soil.

The present results were in line with the findings of Smith and Read [56] in that AM plants frequently produced higher biomass and had higher heavy metal uptake than those without AM fungi. Five main mechanisms can be put forward to explain how AM fungi might increase the adaptability of plants under different soil water regimes: (1): root conductance to water flow may be improved by AM fungi [57]; (2): modification of the amount and distribution of water channel proteins in root membranes for facilitating the passive movement of water molecules down to the water potential gradient [58]; (3): extraradical mycorrhizal hyphae might transport water to colonized roots directly [59]; (4): mycelia of AM fungi may increase soil aggregation [60]; and (5) hyphal aggregation may be formed between soil and roots for preventing air gaps and preserving hydraulic continuity as the soil dried [61,62].

A significant increase in arbuscule richness accompanied by an increase in As uptake by *P. vittata* for soil at 100% WMC indicated the importance of using appropriate AM inoculum for remediation of As contaminated soils. The formation of abuscular in plant root is an important indicator to reflect the restoration process of the contaminated land by plant [63] because the structure can be conclusively established as the transporter of various elements from the surrounding soil to the host plant. A possible explanation could be that plants growing on contaminated soil benefit greatly by arbusculus formation whereby Chu [64] found that such structure can enhance P absorption and activation of P transport system.

4.2. Significance of As species in contaminated soil and the clean up of As by mycorrhizal As hyperaccumulator

The present results showed low concentrations of water-soluble As in all treatments, regardless of influence of water regime. This

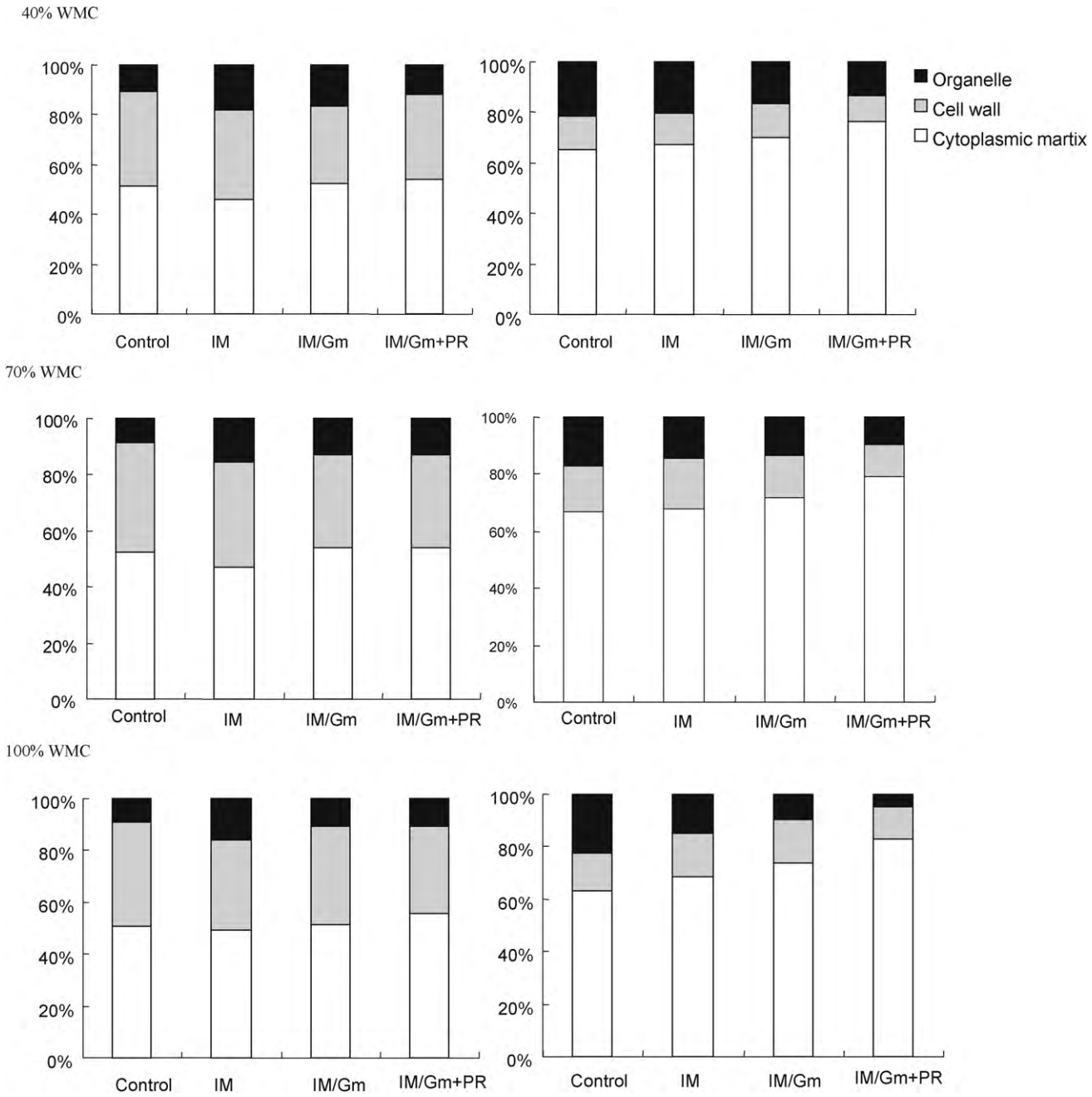

Fig. 5. Subcellular distribution of As in root (left) and frond (right) of *P. vittata* at different WHC of arsenic contaminated soil.
IM = indigenous mycorrhizas in DSL mine site.
IM/Gm = mixed AM inoculum (indigenous mycorrhizas + *G. mosseae*).IM/Gm + PR = mixed AM inoculum + phosphate rock amendment.

is because without stimulation by AM fungi, a low concentration of soluble-form As minerals in soil was formed as a result of low percentage of exchangeable As present in the contaminated soil. Therefore, a higher concentration of unaltered Ca-bounded As containing minerals was dominant in mine soils. The finding was in line with Onken and Adriano [49] who revealed that As became more recalcitrant in Ca-bound fraction which was unavailable for plant uptake. However, the large amount of reduction in Ca–As when treated with IM/Gm + PR implied that *P. vittata* was capable of solubilizing As from the Ca-fraction to other fractions, making it available for plant uptake. Verification of subcellular distribution of As in cell can be achieved by using X-ray microanalysis in future studies.

According to the present study, 23.8% of the initial soil As was removed using mycorrhizal *P. vittata* when treated with IM/Gm + PR at 100% of WMC after 8 weeks. This may have been attributed to the possibility that under a hostile environment (metalloid-contaminated and nutrient-deficient mine soils), the plants may have acquired a specific adaptation capability with the aid of P rock. Such mechanism can enhance the release of As into the soil solution through competitive exchange, resulting in an increase in the bioavailability of As species in soil by P rock [65]. Therefore, AM fungi may assist the host plant in uptaking sufficient P released from P rock, by upregulating low-affinity P transporters located in the mycorrhizal roots [66]. Therefore, elevated As content in plant was found in the experiment.

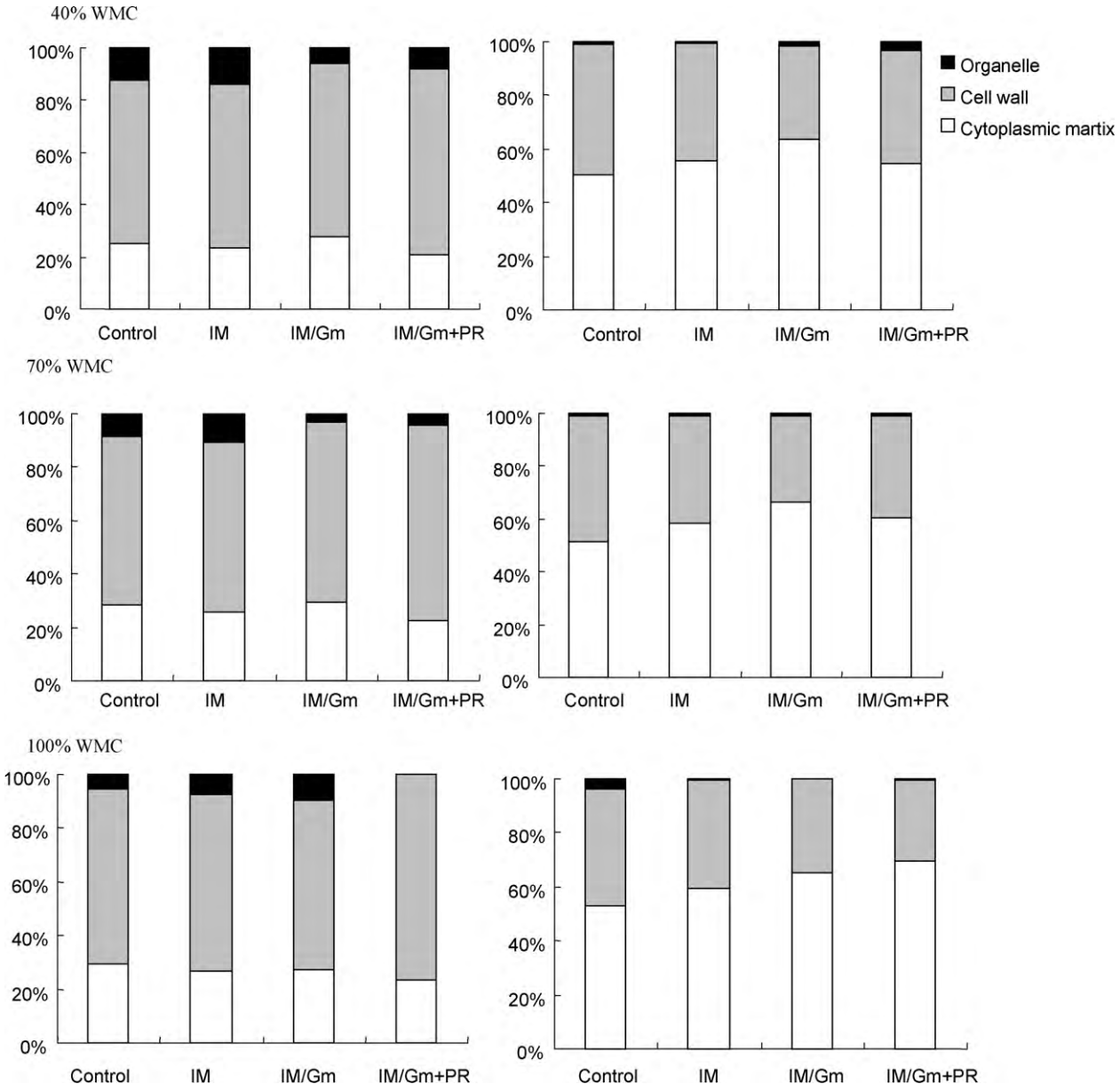

Fig. 6. Subcellular distribution of As in the root (left) and frond (right) of *C. dactylon* at different WHC of arsenic contaminated soil.
IM = indigenous mycorrhizas in DSL mine site.
IM/Gm = mixed AM inoculum (indigenous mycorrhizas + *G. mosseae*).IM/Gm + PR = mixed AM inoculum + phosphate rock amendment.

4.3. Strategies of mycorrhizal plants for As uptake and accumulation

The present results also indicated that As is mainly deposited in cytoplasmic matrix fraction which is composed of cell and vacuole sap. As cell organelle is the main site for cell metabolism, the vacuoles primarily provided a place to store the cellular waste as well as the by-products of cell metabolism [67]. Metal complexes must be stored in vacuoles where they cannot readily dissociate and where they will not interrupt normal metabolic activities of the cell. Thus, most of the absorbed As is eventually enriched within vacuoles whereas As concentration in epidermal cells and organelle are relatively low. Such vacuole compartmentalization may play an important role in the detoxification of heavy metals for hyperaccumulators by buffering the toxic effects of As in contaminated soil.

For non-hyperaccumulator, a higher As concentration was found in the plant cell wall rather than in other cellular parts. The main remediation role played by *C. dactylon* is phytostabilization due to its limited translocation of As from roots to shoots [68]. In addition, the common strategy adopted by the non-hyperaccumulator is to avoid the build-up of excess metal levels in the cytosol by forming entrapment in the apoplast space and chelation of metals using by a range of ligands in plant root [68], in order to prevent the onset of toxicity symptoms.

5. Conclusion

The study indicated that the potential of mixed AM inoculum with an addition of PR improved As uptake in *P. vittata*. The highest percentage of As removal (23.8% of soil As reduction) was found in the hyperaccumulator grown at 100% WHC,

accompanied with the highest plant biomass (17.8 g/pot) and As accumulation (2054 mg/kg DW). Such combination resulted in better root to shoot translocation and subcellular distribution of As in the hyperaccumulator, which is a major criterion for successful phytoextraction. The current study was a short-term greenhouse investigation which demonstrated the significant role of mixed AM inoculum and PR in enhancing plant growth and As hyperaccumulation under adverse conditions especially the stress of higher As concentrations in the growth substrates. However, long-term verification of the results is necessary in order to understand the mechanisms responsible for increased As uptake and translocation in mycorrhizal *P. vittata*, especially in the field. In addition, a recent study revealed that organic carbon and pH are the two important factors that control the availability of As in soils and the level of As uptake and distribution in plant tissues [69]. Further studies should be performed to determine the aggregate benefit of mycorrhizal infection on As hyperaccumulator plant for extracting As from contaminated soil, with different pH and TOC concentration.

Acknowledgements

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