Effectiveness of Applying Arsenate Reducing Bacteria to Enhance Arsenic Removal From Polluted Soils by Pteris Vittata L.

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EFFECTIVENESS OF APPLYING ARSENATE REDUCING BACTERIA TO ENHANCE ARSENIC REMOVAL FROM POLLUTED SOILS BY *PTERIS VITTATA* L.

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Arsenic is a common contaminant in soils and water. It is well established that the fern **Pteris vittata** *L. is an As hyperaccumulator and therefore has potential to phyroremediate As-polluted soils. Also, it is accepted that rhizosphere microflora play an enhancing role in plant uptake of metallic elements from soils. Studies showed that hydroponiclly grown* **P. Vittata** *accumulated arsenite more than the arsenate form of As apparently because arsenate and phosphate are analogues and therefore its absorption is inhibited by phosphate. The objective of this study was to determine whether addition of five different arsenate-reducing bacteria would enhance arsenic uptake by* **P. vittata** *grown in arsenic polluted soils in a field experiment. Results showed that addition of the As reducing bacteria promoted the growth of* **P. vittata***, increased As accumulation, activated soil insoluble As, and reduced As leaching compared to the untreated control. Plant biomass increased by 53% and As uptake by 44%. As leaching was reduced by 29% to 71% depending on the As reducing bacterium. The results in their entirety permitted some insight into the mechanisms by which the arsenate reducing bacteria enhanced the effectiveness of* **P. vittata** *to remove As from the polluted soil.*

KEY WORDS: phytoremediation, soil remediation, soil metal uptake, soil microbial biomass, arsenic leaching

INTRODUCTION

Arsenic (As) and its compounds are widely present in soils and water and cause many human ailments when ingested, including cancer (Tseng et al. 1968; Chen and Ahsan 2004). The threat from As contaminated soil and water has increased with industrialization in many countries. Millions of people in India, Bengal, Australia, South America, and Japan suffer from deleterious health effects from drinking waters polluted by As (Alaerts et al. 2001; Mandal and Suzuki 2002; Ohno et al. 2007). An estimated 200,000 to 270,000 people worldwide have died of cancer caused by drinking As-contaminated water (Gebel 1999; Harvey et al. 2002; Meharg and Rahman 2003). In China As-related diseases from

consumption of As polluted drinking water and foods are present in some areas of Hunan, Guizhou, Xinjiang, Inner Mongolia, Yunnan, Hubei as well as Taiwan (Tseng et al. 1968).

Technologies for remediation of As-contaminated soils and waters have become increasingly important all over the world (Smith et al. 2000; Matschullat 2000; Meharg 2004; Jankong et al. 2007). Chemical and/or physical remediation techniques have been used but are time consuming, costly, and can result in secondary pollution (Huang et al. 2004). By comparison, phytoremediation has the potential to be a less costly and environmentally friendly *in-situ* remediation technology.

The discovery (Ma et al. 2001; Chen et al. 2002) of the first As-hyperaccumulator plant, a fern *Pteris vittata* L. (member of the maidenhair fern family common name Chinese ladder brake), has attracted much attention and generated interest in phytoremediation of As-contaminated soil (Ma et al. 2001; Tu and Ma 2002; Chen et al. 2002; Tu et al. 2004a). However, *P. vittata* grows slowly and is not adapted to a wide range of ecological conditions. These characteristics limit its use to phytoremediate As-contaminated lands over large areas. Studies of ways to promote the growth of *P. vittata* and increase its ability to remove As from contaminated soil would therefore have much practical significance.

The mutually beneficial inter-relationships between plants and soil microbes have been recognized and studied for a long time. The metabolic activity of microbes present in the rhizosphere in natural soils can enhance plant nutrient uptake, promote plant tolerance to pathogens and heavy metals, and increase the synthesis of plant growth factors and degradation of humic compounds. On the other hand, the plant provides the root exudates that increase the soil microbial activity and biomass (Gonzalez-Chavez et al. 2002; Meharg et al. 1994; Sharples et al. 2000; Trotta et al. 2006; Walton and Anderson 1990). Inoculation with rhizobacteria generally produces larger aboveground biomass and alters metal bioavailability in the soil (Wu et al. 2006). Biomass of *P. vittata* is increased after inoculation with arbuscular mycorrhizas (AM) in As-contaminated soil (Leung et al. 2006). Zaidi et al. (2006) also found that AM inoculation also increased biomass in chickpea (*Cicer arietinum* L.).

The ability of plants to take up and accumulate heavy metals in general depends on their genetics, the physical and chemical properties of the soil, the forms of the heavy metals present in the soil, and the composition of the microbial flora (Gonzaga et al. 2007). Rhizosphere microbes impact the chemical interactions between soil and heavy metals such as the absorption and desorption equilibria, the oxidation and reduction reaction, and the mobility and fixation (Robert and Berthelin 1986). More recently it was shown that the bioavailability of soil heavy metals could be influenced by the population variation of rhizosphere microbes, the interactions between microbes and plant roots, and the microbial exudates (Tang et al. 2001). As a result, application of microbes for enhancing phytoremediation has been attempted with positive results in small-scale laboratory studies (Agely et al. 2005; Huang et al. 2004; Liu et al. 2005; Lucy et al. 2004; Vogel-Mikuš et al. 2006). However, little information is available regarding the mechanisms by which microbes enhance the phytoremediation of polluted soils and waters.

It has been shown that *P. Vittata* grown hydroponically utilizes both arsenite and arsenate, but takes up more arsenite. The reason is that arsenate and phosphate are analogues and arsenate absorption is inhibited by phosphate (Wang et al. 2002; Tu et al. 2004b). Based on this study we posed the question whether reducing soil arsenate into arsenite in As-contaminated soil using arsenate reducing bacteria would enhance arsenic uptake by *P. vittata* and thus improve its phytoremediation effectiveness.

The objectives of this study were to (1) determine the effect of soil application of arsenate reducing bacteria on the growth, As uptake, and phytoremediation effectiveness of *P. vittata*, and (2) examine the changes in soil As availability, soil As fractions, and soil microbes caused by adding arsenate reducing bacteria to gain some insight into possible mechanisms for observed effects.

MATERIALS AND METHODS

Experimental Site

The field experiment was conducted in Dengjiatang (N 25°48′ E 113°02′; 335 m above sea level), a township of Chenzhou city, China. The location has a typical subtropical monsoon climate with 19°C annual average temperature, 32.7°C highest monthly average temperature in July, and 5.1°C of lowest monthly average temperature in December. The annual mean rainfall (1996–2002) was 1696 mm (Liao et al. 2004).

The experimental site was in the lower reaches of a valley surrounded by small hills and was severely polluted by As emitted from an As smelting plant in the upper reaches of the valley. The lime soil (Ultisol) had a total As concentration of 83.60 mg kg^{-1}, and the physical and chemical properties were as follows: sand 379.64 g kg^{-1}, silt 327.10 g kg^{-1}, clay 293.26 g kg^{-1} by the Pipette method (Day 1965), pH in a 1:1 soil/water suspension 7.8, total organic matter 22.26 g kg^{-1} by the Walkley-Black method (Nelson and Sommers 1982); total N 0.78 g kg^{-1} by the Kjeldahl method (Bremner and Mulvaney1982); total P 0.56 g kg^{-1} by the antimony-phosphate-molybdate complex method (Olsen and Sommers 1982); total K 9.08 g kg^{-1} by atomic absorption spectrophotometry (AA240FS); available N 100.64 mg kg^{-1} by alkaline hydrolysis method (Bremner and Mulvaney1982); available P of 19.20 mg kg^{-1} by the Olsen method (Bremner and Mulvaney1982); available K 83.00 mg kg^{-1} (ammonium acetate method).

Arsenate-reducing Microbes

The five arsenate reducing bacteria, *Rhodococcus* sp.TS1, *Delftia* sp.TS33, *Comamonas* sp.TS37, *Delftia* sp.TS41, and *Streptomyces lividans* sp. PSQ22, were used. The first 4 were from National Microbiology Laboratory of Huazhong Agricultural University, and the last one was kindly provided by Fudan University. The five bacteria's GenBank Accession Number was EU073067, EU073099, EU073103, EU073107, and AY943951, and the Minimal Inhibitory Concentrations (MIC) in Chemical Defined Medium (CDM) for arsenate resistance were 16 mM, 18 mM, 21 mM, 20 mM, and not determined, respectively.

Experimental Plots, Treatments, and Sampling

Drainage lysimeters 1 m × 1 m and depth of 0.60 m were constructed. There were seven treatments namely: addition of five arsenate reducing bacteria and two controls designated as CK1 (plants with no microbes) and CK2 (without both microbes and plants). Each treatment was replicated four times for a total of 28 lysimeters laid out in a completely randomized design. The lysimeters were filled with well-mixed As-contaminated soil and fertilized with 50.0 g m^{-2} compound fertilizer (15-15-15) applied and to the 0–20 cm depth. They were irrigated regularly using As-free tap water.

Eight 4-month old plants of *P. vittata* with similar size were transplanted in each lysimeter on a 25 cm × 25 cm spacing. One week after transplanting, the five arsenate reducing bacteria were applied evenly as a microbe solution to the soil surface and mixed with the soil to a depth of 20 cm. Soil samples from the upper 20 cm of soil and the percolation water samples were collected after the application of bacteria and at three months. All the samples were kept in refrigerator at 4°C and were analyzed within no more than a week. The fronds of the ferns were harvested after three months, were thoroughly washed with tap water, and then rinsed with deionized water. The fronds were dried in a forced air oven (1 h at 80°C followed by 72 h at 60°C) and weighed. They were ground using a dispersion machine (IKA T18 basic) and stored for chemical analysis.

Chemical Analyses

The soil microbial biomass carbon (SMBC) was extracted by chloroform fumigation-extraction method (Jenkinson and Powlson 1976). Briefly, Pass all soil samples through a 2-mm sieve, adjust water-holding capacity to 45–50%, and pre-incubate at 25°C for 1 week before analysis. The carbon was determined using TOC automatic analyzer (TEKMAR Apollo 9000).

Soil available As was extracted using 0.5 M $NaHCO_3$ at pH 8.5. For total As determination the soils were digested using H_2SO_4-$HClO_4$ and the plants were digested using H_2SO_4-H_2O_2. Arsenic in the lysimeter drainage water was determined after filtration through a 0.45 μm Millipore filter. The total soil As was fractionated into labile As, Al-bound As, Fe-bound As, and Ca-bound As, extracted using NH_4Cl, NH_4F, $NaOH$, and H_2SO_4, respectively (Manful 1992; Herreweghe et al. 2003). The residual As was taken as the difference between the total As and the sum of these fractions. Arsenic in the extracts was determined using hydride generating atomic absorption spectrometry (HG-AA240FS). For quality assurance and quality control (QA/QC) purposes, the standard reference materials for soil (GBW07404) and plants (GBW07603) were obtained from the China National Center for Standard Reference Materials and included in the analysis of the samples.

Statistical Analyses

Statistical analysis of variance for treatment effects were performed using SAS 8.0 software. The Tukey mean separation procedure was used to determine significant differences at a probability level of $P \leq 0.05$.

RESULTS AND DISCUSSION

Treatments with arsenate reducing bacteria increased the dry biomass of fronds by 18% to 50% as compared with the control (CK1) without application of microbes (Table 1). Biomass for treatments with bacteria Ts1, Ts33, and Ts37 was 148–153% that of the CK1 control. Corresponding values for Ts41 and PSQ22 were relatively lower at 118–127% that of the control (Table 2). Since biomass is proportional to the total amount of pollutant accumulation, biomass increase is thus a positive effect of addition of microbes on phytoremediation efficiency (Chen et al. 2002).

The application of arsenate reducing bacteria in four cases significantly increased As concentrations in the fronds of *P. vittata* over the control without microbes (CK1). Increases

Table 1 Dry biomass of *P. vittata* fronds, As concentration and uptake from As-polluted soil treated with arsenate reducing bacteria

Arsenate reducing bacteria	Frond dry biomass g m^{-2}	As concentration mg kg^{-1}	As uptake mg m^{-2}
None (CK1 control)	49.49 c	615.74 c	30.47 c
Ts1	75.94 a	704.32 b	53.39 b
Ts33	73.40 a	886.47 a	65.04 a
Ts37	74.08 a	652.55 bc	48.33 b
Ts41	58.63 bc	835.56 a	48.98 b
PSQ22	63.04 b	698.47 b	44.04 b

Means followed by the same letter in a given column are not significantly different at the 5% level.

ranged from by 6–44%. Treatment with Ts33 produced the highest As concentration of 886.47 mg kg^{-1} and was 144% that the control (615.74 mg kg^{-1}) (Table 1)

Application of microbes may inhibit or enhance plant uptake of heavy metals (Jetiyanon and Kloepper 2002) but from the phytoremediation perspective the enhancement effects are emphasized. Similar findings as those of this study have been reported for removal of nickel contaminated soils by yellowtuft *Alyssum murale* (in the Brassicaceae or mustard family) after addition of *Sphingomonas macrogoltabidus, Microbacterium liquefaciens* and *Microbacterium arabinogalactanolyticum* (Abou-Shanab et al. 2003), and for selenium removal by Indian mustard (*Brassica juncea* L.) in conjunction with *Penicillium* sp (Souza et al. 1999). Zinc uptake by Alpine pennygrass *Thlaspi caerulescens (*Whiting et al. 2001) and As uptake by the silverback fern *Pityrogramma calomelanos* (Jankong et al. 2007) were also enhanced by addition of rhizosphere microbes. However, the mechanisms for these effects are not as well studied and demonstrated as those that increase the uptake of essential nutrients such nitrogen, phosphate and potassium (Siddiqui and Mahmood 2001; Jetiyanon and Kloepper 2002; Raj et al. 2003; Lucas García et al. 2004a, 2004b; Çakmakçi et al. 2006) and underscores the need for more study on the inter-relationships between rhizosphere micro-organisms, plant growth, and uptake of soil chemicals.

As expected, As uptake calculated as the product of As concentration and dry biomass (Table 1) was significantly influenced by application of arsenate reducing bacteria due to the increase of biomass and As concentration of plant. All five microbe treatments had a

Table 2 Fractionation of soil arsenic influenced by application of microbes

Microbes	mg kg^{-1}				
	Labile As	Al-bound As	Fe-bound As	Ca-bound As	Residue As
CK1	0.56 cd	5.27 ab	9.88 c	17.04 ab	42.35 a
CK2	0.72 a	5.77 a	11.29 b	17.34 ab	43.75 a
Ts1	0.52 d	5.40 ab	11.65 ab	15.86 b	34.09 b
Ts33	0.70 ab	4.97 b	10.44 bc	16.68 ab	38.84 ab
Ts37	0.69 abc	4.99 b	10.48 bc	18.26 a	34.52 b
Ts41	0.69 abc	5.36 ab	10.47 bc	16.79 ab	33.33 b
PSQ22	0.58 bcd	5.30 ab	12.86 a	17.74 a	33.25 b

*Data with different letters in the same column indicate significance at $P < 0.05$.

*CK1 was the control without microbe addition, and CK2 was the control without both plants and microbes.
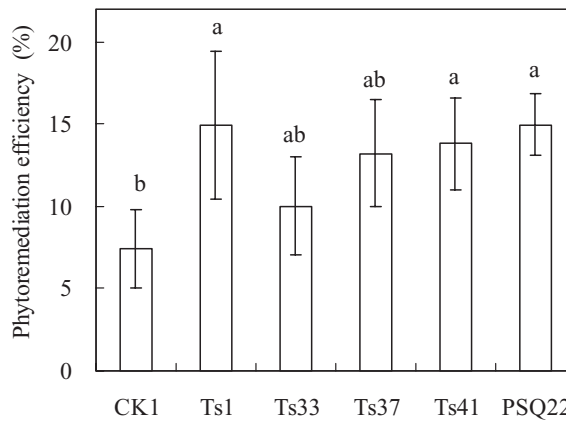
Figure 1 Phytoremediation efficiency of arsenic contaminated soil by *P. vittata* supplied with 5 arsenate reducing bacteria (Ts1, Ts 33, Ts37, Ts41, and PSQ22). CK1 was control without addition of microbes. The phytoremediation efficiency was calculated as 1 minus the ratio of the soil As after 3 months as a percent to the As concentration of original soil. The phytoremediation efficiency percentages were statistically processed by arcsine transformation before variance analysis. Different letters among the pillars indicate significance at $P < 0.05$.

significant increase of As uptake over the control. Increases ranged from 45% for treatment with PSQ22 to 113% for treatment with Ts33 (Table 1).

The phytoremediation efficiency as a percentage was calculated as 1 minus the ratio of the As concentration in the upper 20 cm of the soil after 3 months to the As concentration of original soil. As shown in Figure 1, the phytoremediation efficiency for *P. vittata* was >8% for all treatments. The total As concentrations of soils (0–20 cm) after plant growth for 3 months were significant decreased. Calculation of phytoremediation efficiency for treatment PSQ22 was the highest, and Ts33 the lowest, which were 45% and 17% greater than control, respectively.

One of the basic reasons for the effect of addition of arsenate reducing bacteria would be significant increase of microbial biomass in the rhizosphere of *P. vittata*. Figure 2 showed that addition of arsenate reducing bacteria increased soil microbial biomass carbon (SMBC). For instance, treatment with Ts33 had the highest SMBC, 182.78 mg kg^{-1}, which was approximate 4-fold and 10-fold that of CK1 and CK2, respectively. And even the SMBC of 74.6 mg kg^{-1} for treatment Ts41, the lowest, was approximate 1.7-fold and 3-fold greater than that of CK1 and CK2, respectively.

Generally speaking, heavy metal stress causes the changes of structure, quantity, distribution and even metabolism function of microorganism community in contaminated soils (Frostegard et al. 1996). Under the condition of the long-term heavy metal stress, the biomass amount of micro-organisms in soil decrease significantly (Hiroki 1993; Bardgett et al. 1994). Arsenic contamination caused a sharp decline in the content of microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) in soil. Ghosh et al. (2004) reported that the percentages of MBC/OC (Organic Carbon) and MBN/TN (Total Nitrogen) in an As-contaminated soil decreased by 2 and 3.5 times, respectively, as compared with non-pollution soils. Apparently, these results suggested that increase of soil beneficial microorganism improve soil ecological health, enhance the resistance to heavy metal stress and thus enhance the growth and development of plants.
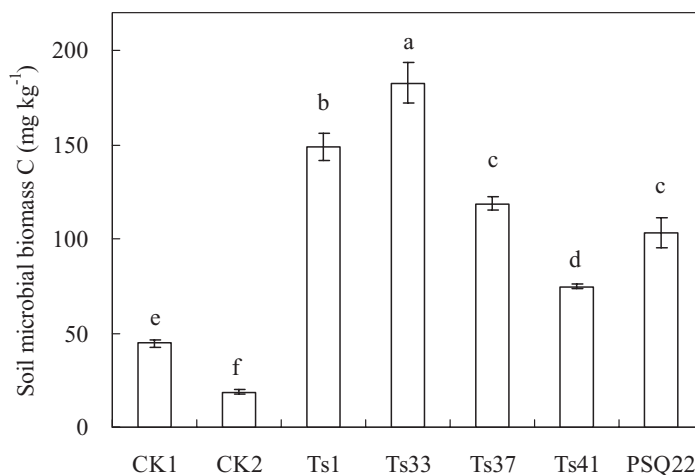
Figure 2 Soil microbial biomass carbon (SMBC) influenced by application of arsenate reducing bacteria after plant growth for 3 months. CK1 and CK2 were two controls that were without microbes and without both microbes and plants, respectively. Different letters among the pillars means significant difference at $P < 0.05$.

Another possible explanation for the effect of addition of arsenates reducing bacteria can be drawn from the results of the analysis of the As forms in the upper 0–20 cm of the soil (Table 2).

Application of microbes decreased the concentrations of soil residual As but slightly increased or had little effect on the concentrations of the available forms of As (labile As, Al bound As, Fe bound As, and Ca bound As), indicating that microbes activated soil insoluble As and thus increased As uptake by *P. vittata* (Table 2). Previous studies found that *P. vittata* take up As from some common arsenic compounds in soil including synthetic $AlAsO_4 \cdot 2H_2O$, $Ca_3 (AsO_4)_2 \cdot 14H_2O$, and $FeAsO_4 \cdot 2H_2O$ (Tu et al. 2004b; Tu and Ma 2002). Also *P. vittata* can effectively take up the two major species of As in soils namely As(III) and As(V). However, as As(V) and PO_4^{3-} are chemical analogues and are transported by the same carrier of plant cells, PO_4^{3-} may interfere or inhibit the uptake of As(V) (Tu and Ma 2003; Fayiga et al. 2008). Thus, arsenate reducing bacteria reduce As(V) into As(III) and might help to increase As uptake and accumulation by *P. vittata*. How efficiently the microbes added in this experiment reduce the As (V) into As(III) are currently being investigated.

This explanation is also supported by the results of the As concentrations in the percolation water from the lysimeter. Long-term monitoring of the rainfall in the lysimeters from June to September showed great amount of seepage flow. Determination of As in percolation water collected during the experiment showed that microbe application all decreased significantly the As leaking by 29% to 71% and 31% to 72% as compared with the two controls (CK1 and CK2), respectively (Figure 3). Addition of Ts37, Ts41, and PSQ22 had the obvious effects, followed by Ts33, and Ts1. The possible explanation was that the application of arsenate reducing bacteria improved the rhizosphere microbial environment, and increased the number and the mycelium of microbes as well as enhanced the biomass of the plant root systems, which might help to take up As, hold soil As, and prevent As losing.
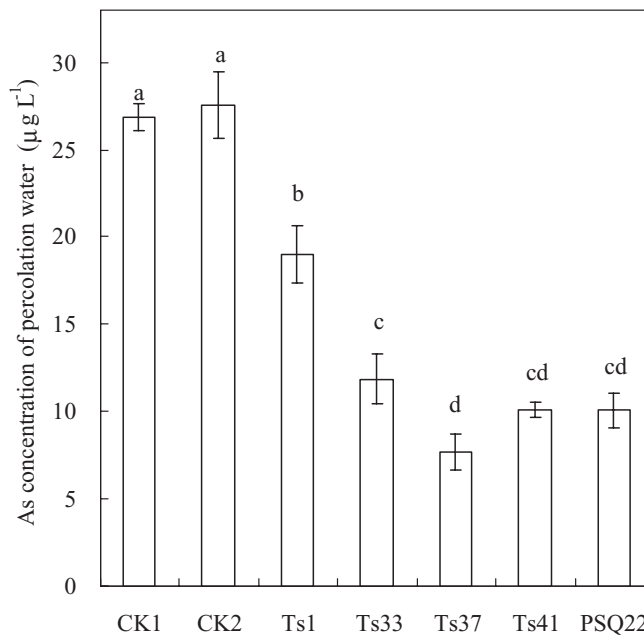
Figure 3 Effects of arsenate reducing bacteria application (Ts1, Ts 33, Ts37, Ts41, and PSQ22) on As concentrations in soil percolating water sampled at 3 month. CK1 was the control without addition of microbes. CK2 was the control without both microbes and plants. Different letters among the pillars means significant difference at $P < 0.05$.

Application of arsenate reducing bacteria increased As bioavailability in soil but not the As concentrations in percolation water (Figure 2) implying reduced possibility of As contamination of groundwater.

CONCLUSIONS

Application of arsenate reducing bacteria significantly promoted growth, As accumulation and remediation efficiency of As polluted soils by *P. vittata* in field conditions. Compared to the control, the bacteria applied treatments markedly enhanced microbial biomass in rhizosphere soil. Furthermore, concentrations of available As including the soil labile As, Fe bound As and Al bound As increased after bacteria was applied, which proved that microbial inoculation increase the bioavailability of As in the soil and thereby improving As uptake by plants. In addition, arsenate reducing bacteria was able to decrease As leaching from the soil, which was of some significance in ensuring the groundwater safety.

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