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INFLUENCE OF ARBUSCULAR MYCORRHIZA AND RHIZOBIUM ON PHYTOREMEDIATION BY ALFALFA OF AN AGRICULTURAL SOIL CONTAMINATED WITH WEATHERED PCBs: A FIELD STUDY

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A field experiment was conducted to study the effects of inoculation with the arbuscular mycorrhizal fungus Glomus caledonium and/or Rhizobium meliloti on phytoremediation of an agricultural soil contaminated with weathered PCBs by alfalfa grown for 180 days. Planting alfalfa (P), alfalfa inoculated with G. caledonium (P+AM), alfalfa inoculated with R. meliloti (P+R), and alfalfa co-inoculated with R. meliloti and G. caledonium (P+AM+R) decreased significantly initial soil PCB concentrations by 8.1, 12.0, 33.8, and 43.5%, respectively. Inoculation with R. meliloti and/or G. caledonium (P+AM+R) increased the yield of alfalfa, and the accumulation of PCBs in the shoots. Soil microbial counts and the carbon utilization ability of the soil microbial community increased when alfalfa was inoculated with R. meliloti and/or G. caledonium. Results of this field study suggest that synergistic interactions between AMF and Rhizobium may have great potential to enhance phytoremediation by alfalfa of an agricultural soil contaminated with weathered PCBs.

KEY WORDS: contaminated soil, polychlorinated biphenyls, phytoremediation, alfalfa, Rhizobia, Arbuscular mycorrhizal fungi

INTRODUCTION

Polychlorinated biphenyls (PCBs) are among the neutral organic compounds of greatest concern as environmental contaminants (McFarland and Clarke, 1989). Soil is an environmental compartment with a large capacity for PCBs and these compounds have long half-lives in soils. Their tendency to accumulate in the food chain makes them a great environmental and human health risk that requires remedial action (Valle *et al.*, 2005; Cousins *et al.*, 1998). Remediation of PCB-contaminated soils has traditionally been carried out

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by incineration and landfill but these approaches have high-energy costs and involve destruction or removal of the soil matrix and are therefore unsuitable for the remediation of agricultural soils.

Microbe-assisted phytoremediation may have some potential as an effective and inexpensive means to clean up polluted soils and has been investigated in laboratory, greenhouse and field conditions (Greenberg, 2006; Gerhardt *et al.*, 2009; Chaudhry *et al.*, 2005; Kuiper *et al.*, 2004; Singer *et al.*, 2004; Huang *et al.*, 2004, 2005; Liste and Alexander, 2000; Banks *et al.*, 2003; Barac *et al.*, 2004; Leigh *et al.*, 2006). It is well known that arbuscular mycorrhizal fungi (AMF) and symbiotic nitrogen-fixing bacteria are common beneficial microbes of monocotyledonous and leguminous plants. The roots of many plant species are colonized by AMF, which subsequently facilitate host plant acquisition of soil phosphorus. In the other key agricultural symbiosis, rhizobia colonize the roots of legumes where they fix atmospheric N₂, some of which can be utilized for plant growth (Marx, 2004). Therefore, environmental biologists have begun to study the potential of these two types of symbiosis to remediate soils contaminated with persistent organic pollutants (POP) such as PCBs, polycyclic aromatic hydrocarbons (PAHs), and petroleum hydrocarbon compounds (PHC) (Chekol *et al.*, 2004; Mehmannavaz *et al.*, 2002; Joner and Leyval, 2003; Radwan *et al.*, 2007). Much of the work has been carried out under laboratory or greenhouse conditions. White *et al.* (2006) conducted field experiments on the effects of mycorrhizal fungi on the phytoremediation of weathered p,p-DDE by *Cucurbita pepo*. However, it is not known whether alfalfa inoculated with rhizobia or arbuscular mycorrhizal fungi has the potential to remediate POP-contaminated field soils.

Legumes can form a tripartite symbiosis with both rhizobia and AMF. The potential importance of the tripartite legume-AMF-*Rhizobium* symbiosis for agriculture and ecology has been recognized and several attempts have been made to find the most effective combinations of AMF and rhizobia (Valdenegro *et al.*, 2001; Requena *et al.*, 2001; Xavier and Germida, 2002; Scheublin *et al.*, 2004). There have been relatively few studies of the remediation effect of legumes inoculated with both AMF and *Rhizobium* in POP-contaminated soil. Abd-Alla *et al.* (2000) reported that pesticides affected plant growth, *Rhizobium/Bradyrhizobium* and AM fungi at different stages of plant growth and effects varied with pesticide and plant species. In a previous pot experiment, we found that alfalfa inoculated with both AMF and *Rhizobium* enhanced the removal of PCB from soil (Teng *et al.*, 2008). However, little is known about the field remediation effect of alfalfa inoculated with AMF and rhizobia in an agricultural soil contaminated with weathered PCBs. The aims of the present study were therefore to further determine whether alfalfa inoculated with AMF and/or *Rhizobium* has a detectable role in the remediation of an agricultural soil contaminated with weathered PCBs in the field and also to explore the shifts in soil microbial populations and microbial community metabolic profiles in the rhizosphere.

MATERIALS AND METHODS

Site Description and Soil Preparation

The study was conducted at a contaminated former transformer and electronic waste stripping and recycling site at Luqiao township, Taizhou city, located at 28°22'N, 121°22'E in Zhejiang Province, east China. In 1989, some arbitrary disposal of used electric appliances resulted in accidental pollution with PCBs. There is no other PCB pollution source near this area, which now has a twenty-year history of soil PCB pollution. Soil samples

were excavated from the top 15 cm of the soil profile and thoroughly mixed using a rotovator and aliquots were then transferred to 20 cement quadrats (inside dimensions of each approximately 1.2 m long $\times$ 1.2 m wide $\times$ 1.0 m deep). Initial total concentrations of 21 PCB congeners in the soil ranged from 556–575 $\mu\text{g kg}^{-1}$. Average properties of the twenty soil samples were as follows: pH 5.80 units, organic carbon 58.2 g kg^{-1} , total N 3.21 g kg^{-1} , total P 1.49 g kg^{-1} , total K 20.4 g kg^{-1} , exchangeable P 83 mg kg^{-1} , and exchangeable K 230 mg kg^{-1} . Soil pH (soil:H₂O ratio 1:2) was measured using a pH meter with a glass electrode. Soil organic C and total N were determined by dichromate oxidation and Kjeldahl digestion and distillation, respectively. Soil total P was digested with persulphate and determined by spectrophotometry. Soil total K was digested with concentrated HF-HClO₄ and exchangeable K was extracted with ammonium acetate and measured by flame emission or absorption. The soil type was classified as Horti Anthrosols according to the WRB soil classification system.

Microbial Inocula and Host Plants

Inoculum of the AM fungus *Glomus caledonium* 90036, isolated from an uncontaminated fluvo-aquic soil in Henan Province, China (Liao *et al.*, 2003), was propagated on sudangrass (*Sorghum sudanense* [Piper] Stapf) grown in a sandy soil for 90 days in a greenhouse. The inoculum, consisting of spores, mycelium, sandy soil and root fragments, was air dried and passed through a 2-mm sieve. For this study, *Rhizobium* cultures were prepared by inoculating 100 ml of yeast extract mannitol broth with a loopful of cells from a stock culture and growing on a rotary shaker (200 rev min⁻¹) at 28°C for 48 h. The *Rhizobium* cultures contained about 1.8×10^8 colony forming units per ml (CFU ml⁻¹). The *Rhizobium* cultures were absorbed in pre-sterilized peat in a 2:1 ratio (wt/vol) of peat and *Rhizobium* culture and thoroughly mixed to produce the *Rhizobium* inoculum. Alfalfa seeds (*Medicago sativa* L.) were purchased from Jiangsu Academy of Agricultural Sciences, China. Before sowing, alfalfa seeds were surface sterilized in a 10% (v/v) solution of hydrogen peroxide for 10 min, rinsed with sterile distilled water and pre-germinated on moist filter paper overnight.

Experimental Design and Sample Collection

The experimental site was established in May 2007. There were five treatments set up in a fully randomized layout of field plots (quadrats) with each treatment in quadruplicate. The five treatments were: 1) soil with neither planting of alfalfa nor inoculation with *G. caledonium* or *R. meliloti* as unplanted control (CK); 2) uninoculated alfalfa (P); 3) alfalfa inoculated with *G. caledonium* (P+AM); 4) alfalfa inoculated with *R. meliloti* (P+R); 5) alfalfa inoculated with *G. caledonium* and *R. meliloti* (P+AM+R). Surface sterilized and germinated seeds were sown in the cement quadrats in the field and inoculated with 10 g *R. meliloti* and/or 10 g *G. caledonium* inoculum according to the different treatments. Uninoculated treatments received sterilized inoculum. After germination, the seedlings were thinned to twenty-five per quadrat and the plants grew for 180 days. Throughout the growing season, the plants were monitored daily and watered as necessary. Once a week plant growth was measured and weeds were cleared from the quadrats. At the end of the experiment, the rhizosphere soil—defined as the soil firmly adhering to the roots—was removed manually and transferred to a paper envelope (Grayston *et al.*, 1998). Two-hundred grams of composite rhizosphere soil samples were placed in brown glass containers and

stored in a freezer at -80°C prior to extraction. Soil samples for bacterial and fungal counts and determination of microbial community functional diversity were placed in sterile paper bags and stored at 4°C prior to the microbial assays. In addition, five plants were harvested from each quadrat, separated into roots and shoots, and carefully rinsed with distilled water to remove any remaining soil and dust particles.

Analytical Procedures for PCBs in Soil and Plant Samples

Soil samples were thoroughly homogenized, freeze-dried and sieved with a 100-mesh sieve prior to analysis. Soil PCB extractions and analysis were performed using methods described by Gao *et al.*, (2006). Briefly, ten-gram sub-samples were thoroughly mixed and ground with anhydrous sodium sulfate powder, soaked with 40 ml hexane/acetone (1:1 v/v) overnight, sonicated for 1 h, and extracted using a Model KQ-600B sonicator (Kunshan Ultrasonic Instruments). Extraction and ultrasonication were repeated twice, using 20 ml of the same solvent mixture each time. The extracts were combined and collected in 120 ml glass vials with Teflon caps. Extract volumes were condensed to 10 ml using a rotary evaporator. Each extract was then cleaned with concentrated sulfuric acid, washed with saturated Na_2SO_4 solvent, and transferred to a 25×1 cm silica gel column with 1 g anhydrous Na_2SO_4 placed at the top and eluted with 40 ml of n-hexane. The eluate was concentrated and dissolved in 5 ml of n-hexane and 2 ml of the eluate was transferred to an auto-sampler vial before analysis.

Plant samples were carefully chopped with metal scissors and their fresh weight was recorded. Samples were then dried in an oven at 40°C , and then their dry weight was recorded. Approximately 2 g of dry plant sample was then ground with anhydrous sodium sulfate using a ceramic mortar and pestle and extracted as described above. After each sample was ground all equipment was rinsed with acetone in order to minimize cross contamination.

Analysis of soil and plant extracts was conducted on a Varian 3800 GC equipped with an electron capture detector (ECD) and auto injector. A Varian CP-Sil 24CB (length 30 m, i.d. 0.25 mm, film thickness $0.50\ \mu\text{m}$) was used. The carrier gas was N_2 . A $1\text{-}\mu\text{l}$ sample extract was injected. The injector and detector temperatures were 260 and 300°C , respectively. The oven temperature was initially set at 120°C for 0.5 min, ramped at $10^{\circ}\text{C min}^{-1}$ to 180°C , held for 1 min then at $15^{\circ}\text{C min}^{-1}$ to 250°C , and held for 25 min. PCBs were identified by retention time matches to standards and were quantified using peak area integration. All the samples were analyzed in triplicate. Certified standards in iso-octane (J&K Company: 21 PCB congeners comprising PCB8, PCB 18, PCB 28, PCB 44, PCB 52, PCB 66, PCB 77, PCB 101, PCB 105, PCB 118, PCB 126, PCB 128, PCB 138, PCB 153, PCB 170, PCB 180, PCB 187, PCB 195, PCB 200, PCB 206, and PCB 209) were used to prepare standard curves for gas chromatography. Concentrations of PCBs were quantified by an external standard and all the results are expressed on a dry weight basis. The recoveries of PCBs from spiked samples were in the range of 76.3–98.5%. The detection limits ranged from $1.33\text{--}3.45\ \mu\text{g kg}^{-1}$, with a linear range from $0.5\text{--}50\ \text{ng ml}^{-1}$. The coefficient of determination was 0.9995.

Assessment of AM Colonization of Alfalfa Roots

The percentage of AMF-colonized root length was determined using a modification of the gridline intersect method of Giovannetti and Mosse (1980). Briefly, a one-gram

aliquot of fresh roots was cut into short segments (3–5 mm) and the root segments were cleared in 10% KOH for 10 min at 90°C in a water bath, rinsed in water, and then stained with 0.1% Trypan blue for 3–5 min at 90°C in a water bath. The percentage of root length colonized by mycorrhiza was assessed by visual observation of fungal colonisation and estimated using the grid-line intersect method.

Soil Microbial Counts and Microbial Community Metabolic Profiles

Counts of cultivable bacteria and fungi were performed using a standard dilution-plating procedure on various agar media (Larkin *et al.*, 2003). Ten grams of soil sample were suspended in 90 ml of sterile water and shaken for 20 min. Then ten-fold serial dilutions were plated on the different selective media. Data are expressed as number of colony forming units (CFU) per gram of dry soil. Biphenyl-degrading bacteria were counted in 96-well microplates with five replicates per dilution by the most probable number (MPN) method using a basal mineral medium without carbon but supplemented with biphenyl to yield a concentration of 50 mg ml⁻¹ in the liquid phase (Wagner-Döbler *et al.*, 1998).

Analysis of soil microbial community metabolic profiles was performed as described by Campbell *et al.* (1997) and Yao *et al.* (2000). Briefly, 10 g of fresh soil were added to 100 ml of distilled water in a 250-ml flask and shaken for 10 min. Ten-fold serial dilutions were made and the 10⁻³ dilution was used to inoculate the Biolog GN plates. Plates were incubated at 25°C for seven day, and color development was measured as absorbance (A) using an automated plate reader (VMAX, Molecular Devices, Crawley, UK) at 590 nm and the data were collected using Microlog 4.01 software (Biolog, Hayward, CA, USA). Average well color development (AWCD) was calculated according to Garland and Mills (1991), i.e., $AWCD = \Sigma(C-R)/n$ where C is color production within each well (optical density measurement), R is the absorbance value of the plate's control well, and n is the number of substrates. Mean AWCD values were plotted over time for each sample.

Statistical Analysis

Statistical analysis was carried out using the SPSS 13.0 for Windows software package. The chemical and microbiological data were analysed by one-way analysis of variance. Treatment means were compared using Duncan's multiple range test.

RESULTS

Plant Biomass, Nodule Weight, and Mycorrhizal Colonization

Plant shoot and root dry biomass, root nodule weight and mycorrhizal colonization in the different treatments are presented in Table 1. Inoculation with *R. meliloti* alone had a greater effect than *G. caledonium* alone on the yield parameters. When alfalfa was inoculated with both *R. meliloti* and *G. caledonium* symbionts, shoot biomass, root dry biomass and root nodule dry weight were significantly greater compared with those inoculated with only *G. caledonium* or uninoculated plants ($p < 0.05$). There was no significant effect of dual inoculation with *R. meliloti* and *G. caledonium* symbionts on the alfalfa biomass or root nodule weight when compared with those inoculated with *R. meliloti* alone ($p > 0.05$). Root mycorrhizal colonization increased significantly in the presence of *R. meliloti* (Table 1) and was significantly greater in plants with dual inoculation with *R. meliloti* and *G. caledonium*

Table 1 Plant dry biomass, root nodule dry weight and mycorrhizal colonization of P (uninoculated alfalfa), P+AM (alfalfa inoculated with *Glomus caledonium*), P+R (alfalfa inoculated with *Rhizobium meliloti*), and P+AM+R (alfalfa co-inoculated with *Rhizobium meliloti* and *Glomus caledonium*)

Component	P	P+AM	P+R	P+AM+R
Shoot biomass (g plant ⁻¹)	135 ± 23 ^b	149 ± 26 ^b	241 ± 30 ^a	266 ± 36 ^a
Root biomass (g plant ⁻¹)	109 ± 13 ^b	112 ± 14 ^b	156 ± 19 ^a	168 ± 22 ^a
Root nodules (g plant ⁻¹)	2.88 ± 0.57 ^b	3.22 ± 0.62 ^b	5.98 ± 0.73 ^a	6.64 ± 0.88 ^a
Mycorrhizal colonization (%)	19.1 ± 1.25 ^c	79.4 ± 4.12 ^b	21.8 ± 1.69 ^c	84.3 ± 2.35 ^a

Values are means ± standard deviation. Different letters in the same row indicate a significant difference at $p < 0.05$ according to Duncan's multiple range test ($n = 4$).

(P+AM+R) when compared with all the other treatments ($p < 0.05$). Uninoculated alfalfa (P) was colonized by indigenous AMF but the length of root colonized was much smaller than in inoculated treatments.

Soil PCB Concentrations and Congener Profiles

Soil target PCB concentrations after planting with alfalfa and inoculation with AMF and *Rhizobium* are presented in Figure 1. After 180 days of plant growth, the final PCB concentrations across all treatments ranged from 325.4–541.2 $\mu\text{g kg}^{-1}$ dry soil and planting with alfalfa significantly reduced soil PCB concentrations compared with the unplanted

Figure 1 Soil concentrations of target PCBs in the different treatments. CK, control without any planting; P, uninoculated alfalfa; P+AM, alfalfa inoculated with *G. caledonium*; P+R, alfalfa inoculated with *R. meliloti*; P+AM+R, alfalfa inoculated with both *G. caledonium* and *R. meliloti*. Values are means and bars denote standard deviation. Different letters in the same histogram indicate a significant difference at $p < 0.05$ according to Duncan's multiple range test ($n = 4$).

Figure 2 Mean percentage composition of polychlorinated biphenyl homologues in the soil under the different treatments. CB, chlorinated biphenyls; CK, control without planting; P, uninoculated alfalfa; P+AM, alfalfa inoculated with *G. caledonium*; P+R, alfalfa inoculated with *R. meliloti*; P+AM+R, alfalfa inoculated with both *G. caledonium* and *R. meliloti*.

control ($541.2 \mu\text{g kg}^{-1}$ dry soil) ($p < 0.05$). Soil PCB concentrations were lowest in the dual inoculation treatment (P+AM+R) in which they were significantly lower than in all other treatments except inoculation with *R. meliloti* alone. The mean percent composition of polychlorinated biphenyl homologues in the soil under the different treatments are shown in Figure 2. Planting with alfalfa and inoculation with *R. meliloti* or *G. caledonium* enhanced depletion of the lower chlorinated tetra-chlorinated biphenyls and increased the proportion of more highly chlorinated penta- and hexa-chlorinated biphenyls compared with the average congener profile of the unplanted control soil.

PCB Concentrations and Accumulation in Alfalfa Shoots and Roots

Alfalfa inoculated with both *R. meliloti* and *G. caledonium* (P+AM+R) exhibited the highest PCB concentrations in shoots, followed by alfalfa inoculated with *R. meliloti* (P+R), inoculated with *G. caledonium* (P+AM) and uninoculated alfalfa (P) (Figure 3A). The average PCB concentrations in shoots of the P+AM+R, P+R, P+AM, and P treatment were 46.9 , 42.1 , 36.6 , and $27.4 \mu\text{g kg}^{-1}$ dry biomass, respectively. The average PCB concentration in shoots of the P+AM+R treatment was significantly higher than those of both the P+AM and P treatments ($p < 0.05$), but no significant differences were observed

Figure 3 Concentrations of polychlorinated biphenyls in shoots (A) and roots (B) of alfalfa in the different planted treatments. P, uninoculated alfalfa; P+AM, alfalfa inoculated with *G. caledonium*; P+R, alfalfa inoculated with *R. meliloti*; P+AM+R, alfalfa inoculated with both *G. caledonium* and *R. meliloti*. Values are means $\pm$ standard deviation. Different letters in the same histogram indicate a significant difference at $p < 0.05$ according to Duncan's multiple range test ($n = 4$).

in PCB concentration between the P+AM+R and P+R treatments ($p > 0.05$). In roots (Figure 3B) the average PCB concentrations for the P+AM+R, P+R, P+AM, and P treatments were 267.8, 326.1, 324.5, 230.8 $\mu\text{g kg}^{-1}$ dry biomass, respectively. The average PCB concentrations in roots of the P+AM and P+R treatments were significantly higher than in the P treatment ($p < 0.05$), but the P+AM+R treatment showed no significant difference in root PCB concentration compared with either the P+R or the P+AM treatment ($p > 0.05$).

Shoot PCB contents were significantly enhanced in plants inoculated with *R. meliloti* alone (P+R) and inoculated with *R. meliloti* and *G. caledonium* together (P+AM+R) (Figure 4A). Dual inoculation led to a 3.39-fold increase in PCB shoot content compared with the uninoculated alfalfa (P). Greater root PCB contents were observed when alfalfa

Figure 4 Content of polychlorinated biphenyls in shoots (A) and roots (B) of one alfalfa plant in the different planted treatments. P, uninoculated alfalfa; P+AM, alfalfa inoculated with *G. caledonium*; P+R, alfalfa inoculated with *R. meliloti*; P+AM+R, alfalfa inoculated with both *G. caledonium* and *R. meliloti*. Values are means $\pm$ standard deviation. Different letters in the same histogram indicate a significant difference at $p < 0.05$ according to Duncan's multiple range test ($n = 4$).

was inoculated with *R. meliloti* and/or *G. caledonium* (Figure 4B). *R. meliloti* and/or *G. caledonium* inoculation also appeared to have a positive effect on root PCB acquisition.

Bioconcentration Factor (BCF) and Transfer Factor (TF) of PCBs in Alfalfa

Bioconcentration factors (BCF) and transfer factors (TF) can be calculated to evaluate plants for phytoextraction of polychlorinated biphenyls. BCF is defined as the PCB concentration in shoot and root tissues divided by the PCB concentration in the soil. For all planted treatments, the shoot and root BCFs ranged from 0.053–0.144 and 0.447–0.823, respectively (Figure 5A,B). Alfalfa inoculated with *R. meliloti* alone significantly increased the shoot BCF by 111% and the root BCF by 94% compared with uninoculated alfalfa. This significant increase in shoot BCF was greater in dual inoculation with *Rhizobium* and *Glomus* (P+AM+R), with an increase of 172% compared with uninoculated alfalfa (Figure 5A). TF is calculated as the PCB concentration in shoots divided by that in the roots and denotes the ability of PCBs to transfer from roots to shoots. Across all planted treatments the alfalfa TF ranged from 0.119 to 0.175 (Figure 5C). TF was significantly higher in alfalfa inoculated with both *Rhizobium* and *Glomus* (P+AM+R) compared with uninoculated (P) plants and those inoculated with *G. caledonium* (P+AM). There was no significant effect of inoculation with *G. caledonium* alone on the alfalfa TF.

Percent Mass Removal and Phytoextracted of PCBs from Soils

The effects of *R. meliloti* and/or *G. caledonium* inoculation on percent mass removal and phytoextraction of PCBs from soils planted with alfalfa are shown in Table 2. The volume of soil in each quadrat of 120 × 120 × 90 cm multiplied by the mean soil density of 1.18 g cm⁻³ gives a soil mass of 1630 kg, and this value can be used to calculate the absolute quantity of PCBs in the soil quadrats (Table 2). The percent removal of target PCBs by the uninoculated alfalfa (P), alfalfa inoculated with *G. caledonium* (P+AM), alfalfa inoculated with *R. meliloti* (P+R) and alfalfa co-inoculated with *Rhizobium* and *Glomus* (P+AM+R) was 8.1, 12.0, 33.8, and 43.5%, respectively. In the control soils (CK), only 2.7% of the initial PCB levels was observed. For the uninoculated alfalfa PCB the content (mg quadrat⁻¹) and percent phytoextraction by alfalfa in a quadrat were only 0.72 and 0.09%, respectively. However, *R. meliloti* and/or *G. caledonium* inoculation increased PCB uptake and phytoextraction. For example, the percentage of PCBs phytoextracted by alfalfa inoculated with *Rhizobium* was 0.25%, a 2.78 times increase when compared with the uninoculated alfalfa.

Soil Microbial Counts and Microbial Community Metabolic Profiles

Soil culturable bacterial, fungal, and biphenyl-degrading bacterial counts from the different treatments are presented in Table 3. Counts of culturable bacteria, fungi, and biphenyl degrading bacteria in the soils were higher from all the planted treatments than from the unplanted control. Alfalfa inoculated with both symbionts (P+AM+R) and *R. meliloti* alone (P+R) significantly increased the bacterial counts and the biphenyl degrading bacterial counts in the soil ($p < 0.05$) but there was no significant difference between the P+AM+R and P+R treatments. Inoculation with *Glomus* did not show any significant

Figure 5 Bioconcentration factors (BCF) of polychlorinated biphenyls in shoots (A) and roots (B) and transfer factors (TF) (C) of polychlorinated biphenyls in alfalfa in the different planted treatments. P, uninoculated alfalfa; P+AM, alfalfa inoculated with *G. caledonium*; P+R, alfalfa inoculated with *R. meliloti*; P+AM+R, alfalfa inoculated with both *G. caledonium* and *R. meliloti*. Values are means $\pm$ standard deviation. Different letters in the same histogram indicate a significant difference at $p < 0.05$ according to Duncan's multiple range test ($n = 4$).

increase in counts of bacteria or biphenyl degrading bacteria compared with uninoculated plants.

Differences in average well color development (AWCD) in the rhizosphere of alfalfa inoculated with *Glomus* and/or *Rhizobium* are shown in Figure 6. After 120 h, AWCD values significantly discriminated among the soil microbial community metabolic profiles in the

Table 2 Percent mass removal and phytoextraction of PCBs from soils in field quadrats by different treatments. P (uninoculated alfalfa), P+AM (alfalfa inoculated with *G. caledonium*), P+R (alfalfa inoculated with *R. meliloti*), and P+AM+R (alfalfa co-inoculated with *R. meliloti* and *G. caledonium*)

Item	CK	P	P+AM	P+R	P+AM+R
PCB content in initial soil (mg quadrat ⁻¹)	907	916	889	926	938
PCB content in final soil (mg quadrat ⁻¹)	882	842	782	613	530
PCB percent mass removal (%) ^a	2.7	8.1	12.0	33.8	43.5
PCB content in shoots (mg quadrat ⁻¹)	—	0.09	0.14	0.25	0.31
PCB content in roots (mg quadrat ⁻¹)	—	0.63	0.91	1.27	1.12
PCB content in total plant (mg quadrat ⁻¹)	—	0.72	1.04	1.52	1.43
Percentage of PCBs phytoextracted (%) ^b	—	0.09	0.13	0.25	0.27

^aRatio of soil PCB mass removal content to PCB initial content in soil in quadrat.
^bRatio of total PCB mass in alfalfa to that in the estimated 1630 kg of soil per quadrat.

Table 3 Soil culturable bacterial, fungal, and biphenyl-degrading bacterial counts (log CFU or MPN g⁻¹ soil) from the different treatments

Treatment	Bacterial count	Fungal count	Biphenyl degrading bacterial count
Control	7.13 ± 0.11c	6.55 ± 0.28c	6.19 ± 0.13c
P	8.26 ± 0.17b	6.53 ± 0.14c	6.66 ± 0.22b
P+AM	8.35 ± 0.19b	6.92 ± 0.13b	6.89 ± 0.12b
P+R	9.29 ± 0.14a	7.15 ± 0.18ab	7.41 ± 0.28a
P+AM+R	9.35 ± 0.22a	7.34 ± 0.23a	7.48 ± 0.28a

Values are means ± standard deviation. Different letters in the same row indicate a significant difference at $p < 0.05$ according to Duncan's multiple range test ($n = 4$).

Figure 6 Variation in average well color development (AWCD) for soil samples from the different treatments. For explanation of symbols, see Figure 1.

five treatments. Dual inoculation with *Rhizobium* and *Glomus* (P+AM+R) showed the highest AWCD in rhizosphere soil of alfalfa, the P+R treatment exhibited the second highest AWCD, and values for the P+AM treatment and P treatment were intermediate. Moreover, the microbial community metabolic profiles from the P+AM+R, P+R, P+AM and P treatments showed significantly greater carbon utilisation than those from the unplanted control (CK) ($p < 0.05$).

DISCUSSION

Phytoremediation has now emerged as a promising strategy for in situ removal of numerous soil contaminants (Susarla *et al.*, 2002; Macek *et al.*, 2000; Cunningham *et al.*, 1996; Pilon-Smits, 2005; Åslund *et al.*, 2007). Microbe-assisted remediation is a specific type of phytoremediation, which appears to be particularly effective for the removal and/or degradation of organic contaminants from aged soils (Gerhardt *et al.*, 2009). Other studies have also investigated microbe-assisted phytoremediation (Greenberg, 2006; Chaudhry *et al.*, 2005; Kuiper *et al.*, 2004; Singer *et al.*, 2004; Huang *et al.*, 2004, 2005; Banks *et al.*, 2003; Leigh *et al.*, 2006) but there is a need for more information from field conditions. In the present study, planted soils did show significantly lower concentrations of extractable PCBs compared with the unplanted control soil (Figure 1). This suggests that alfalfa in this experiment played a role in the remediation of PCB contaminated soil. Planting with alfalfa inoculated with *G. caledonium* or *R. meliloti* also enhanced the depletion of the lower chlorinated tetra-chlorinated biphenyls (Figure 2). Certain lower-molecular-weight organic compounds may be translocated by plants, stored in cell walls or lignin and lost by transpiration from the vegetative plant parts (Aitchison *et al.*, 2000) or may be degraded within the plant tissues (Newman *et al.*, 1997). Microbial consortia including indigenous and introduced microorganisms in the rhizosphere can work in tandem to effectively enhance the breakdown of organic pollutants in the environment (Macek *et al.*, 2000; Chaudhry *et al.*, 2005; Kuiper *et al.*, 2004; Yateem *et al.*, 2007).

Mycorrhizal associations, and particularly the arbuscular types, can be applied to enhance the phytoremediation of organics (Meharg and Cairney, 2000; White *et al.*, 2006), and some results have shown that these associations may be crucial for both plant establishment on contaminated sites (Leyval and Binet, 1998) and for degradation of PAHs (Joner *et al.*, 2001; Joner and Leyval, 2003). In the present study alfalfa inoculated with *G. caledonium* greatly enhanced the removal of PCBs from soil (Figure 1 and Table 2) and this supports the results of our earlier pot experiments (Teng *et al.*, 2008). In addition, there were higher fungal counts and carbon utilisation ability of the microbial community in rhizosphere soil inoculated with *G. caledonium* compared with uninoculated rhizosphere soil (Table 3 and Figure 6). This may perhaps be explained by effects of mycorrhiza on root physiology (such as enzyme activities and root exudation) that stimulate PCB degradation and changes in the numbers and activities of other rhizosphere microorganisms. Indeed, mycorrhizas enhance the level of hydrogen peroxide in roots (Salzer *et al.*, 1999) and the activities of oxidative enzymes in roots and rhizosphere soil (Criquet *et al.*, 2000) and this may lead to enhanced oxidation of PCBs in the vicinity of mycorrhizal roots. Some studies have also indicated that mycorrhizal colonization results in quantitative and qualitative changes in root exudation, which may in turn modify the microbial community colonizing the rhizosphere of mycorrhizal roots (Graham *et al.*, 1981; Laheurte *et al.*, 1990; Meyer and Linderman, 1986; Wamberg *et al.*, 2003). Mycorrhizas may enhance the degradation of

aromatic compounds in rhizosphere soil or roots and induce the degradation of more complex compounds with comparable chemical structure (Marschner *et al.*, 2001; Chen *et al.*, 1999). Thus, inoculation with *G. caledonium* may increase fungal counts and the carbon utilisation ability of the microbial community that may then degrade PCBs in rhizosphere soil.

In the present study, there were significantly lower concentrations of soil PCBs in the planted treatment inoculated with *R. meliloti* than in the uninoculated planted treatment (Figure 1). Moreover, shoot and root biomass and root nodule dry weight of alfalfa inoculated with *Rhizobium* (P+R) were greater than in the uninoculated alfalfa (Table 1), and correspondingly higher accumulation of PCBs in shoots and roots of alfalfa was observed (Figures 3 and 4). This may be related to the ability of the rhizobial symbiosis to alleviate nutrient limitations, and thus, increase plant and root growth, as well as to enhance the phytoextraction of PCBs in contaminated soils (Table 2). The counts of soil bacteria, fungi and biphenyl-degrading bacteria and carbon utilisation ability of the microbial community in the rhizosphere of alfalfa inoculated with *R. meliloti* all increased significantly compared with the uninoculated planted treatment (Table 2 and Figure 4). Accordingly, we can speculate that after inoculation with *R. meliloti* the dissipation of soil PCBs may be a result of the combination of direct plant uptake or root absorption and biodegradation by the rhizosphere microbial community including rhizobia. Phillips and Streit (1996) found that rhizobia can increase exudation from host roots. Peters *et al.* (1986) found that a plant flavone, luteolin, is a normal secondary plant metabolite found throughout the plant kingdom that may serve to control *nodABC* expression during nodule development. A study by Leigh *et al.* (2002) demonstrated that seasonal fine root death releases several flavones, which act as substrates for PCB degrading bacteria. Thus, increased amounts of exudates may in turn support the growth of microbial degraders or influence pollutant availability. Johnson *et al.* (2004, 2005) also found that symbiotic association with *R. leguminosarum* *bv. trifolii* improved plant vigor and growth in inoculated planted treatments, and that rhizobia play an important role in the rhizoremediation of high-molecular-weight PAHs. It therefore follows that improvement in root growth through the use of a growth stimulating rhizobial inoculum may lead to stimulation of the growth of biphenyl-degrading bacteria. In order to further verify this interesting result, cloning and sequencing analysis of soil DNA found that BphA genes commonly exist in PCB-contaminated soils and were associated with organisms such as *Pseudomonas* sp., *Burkholderia* sp., *Bordetella* sp., and bacteria that could not be cultured. Moreover, the quantity of the functional gene in soil may be correlated with soil biphenyl-degrading bacterial counts (unpublished data). Previous results have shown that rhizobia have the ability to biotransform PCB, either as free-living cells or in symbiosis with host plants (Damaj and Ahmad, 1996; Ahmad *et al.*, 1997; Mehmannaavaz *et al.*, 2002). Our other experiment showed that the degradation rate of *R. meliloti* to 2,4,4'-Trichlorobiphrnyl (PCB28) was 98.5% in the presence of 50 mg L⁻¹ of 2,4,4'-Trichlorobiphrnyl for 7 days. Furthermore, the degradation rate of 18 polychlorinated biphenyl congeners was 54.7%, and in addition, there were microbial transformation processes from higher chlorinated PCBs congeners to lower chlorinated PCBs congeners (unpublished data). Thus, plant-rhizobacterial associations may offer a good remediation strategy for phytoremediation of PCB-contaminated soils.

Compared with the *Rhizobium*-legume symbiosis or AMF-legume symbiosis, the tripartite legume-AMF-*Rhizobium* symbiosis is less understood with respect to its role in the bioremediation of PCB contaminated soils. Our results indicate that there were synergistic interactions between *R. meliloti* and *G. caledonium*, which were manifested as

increases in soil PCB dissipation and shoot biomass of alfalfa compared with uninoculated alfalfa. This could be due to a contribution to improved plant nutrition by the external hyphae of the mycorrhizal fungus and root nodule formation. We also found that alfalfa inoculated with both symionts showed higher plant biomass, root nodule weight, and mycorrhizal colonization levels than the uninoculated alfalfa. Other studies suggest that increased growth, yield, and AMF colonization levels of legumes inoculated with AMF and rhizobia are generally associated with enhanced N and/or P uptake (Manjunath *et al.*, 1984; Pacovsky *et al.*, 1986; Azcón *et al.*, 1991; Ruiz-Lozano and Azcón, 1993; Xavier and Germida, 2002). On the other hand, when alfalfa was inoculated with both symbionts, soil PCB dissipation may have been associated with changes in microbial populations and community structure in the rhizosphere. Biró *et al.* (2000) also reported that there was a further enhancement (a synergistic effect) between *Azospirillum* and *Rhizobium* nitrogen-fixers and arbuscular mycorrhizal fungi in the rhizosphere of alfalfa. Taken together, studies to date suggest that the tripartite alfalfa-*G. caledonium*-*R. meliloti* symbiosis may be a viable bioremediation strategy for agricultural soils contaminated with weathered PCBs.

In conclusion, alfalfa can play a significant role in the dissipation of PCBs in soil and the inoculation with *G. caledonium* and/or *R. meliloti* may have a synergistic effect on phytoremediation of weathered PCBs by alfalfa. The tripartite symbiotic association may therefore offer a very useful remediation strategy for agricultural soils contaminated with weathered PCBs. However, further studies are needed to investigate the metabolic pathway of PCB degradation in plant-microbial associations and the molecular feedback mechanisms that lead to PCB degradation and transformation in the rhizosphere.

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