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RESEARCH ARTICLE

Bioremediation of Cd and carbendazim co-contaminated soil by Cd-hyperaccumulator *Sedum alfredii* associated with carbendazim-degrading bacterial strains

Wendan Xiao · Huan Wang · Tingqiang Li · Zhiqiang Zhu · Jie Zhang · Zhenli He · Xiaoe Yang

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Abstract The objective of this study was to develop a bioremediation strategy for cadmium (Cd) and carbendazim co-contaminated soil using a hyperaccumulator plant (*Sedum alfredii*) combined with carbendazim-degrading bacterial strains (*Bacillus subtilis*, *Paracoccus* sp., *Flavobacterium* and *Pseudomonas* sp.). A pot experiment was conducted under greenhouse conditions for 180 days with *S. alfredii* and/or carbendazim-degrading strains grown in soil artificially polluted with two levels of contaminants (low level, 1 mg kg^{-1} Cd and 21 mg kg^{-1} carbendazim; high level, 6 mg kg^{-1} Cd and 117 mg kg^{-1} carbendazim). Cd removal efficiencies were 32.3–35.1 % and 7.8–8.2 % for the low and high contaminant level, respectively. Inoculation with carbendazim-degrading bacterial strains significantly ($P<0.05$) increased Cd removal efficiencies at the low level. The carbendazim removal efficiencies increased by 32.1–42.5 % by the association of *S. alfredii* with carbendazim-degrading bacterial strains, as compared to control, regardless of contaminant level. Cultivation with *S. alfredii* and inoculation of carbendazim-degrading bacterial strains increased soil microbial biomass, dehydrogenase activities and microbial diversities by 46.2–121.3 %, 64.2–143.4 %, and 2.4–24.7 %, respectively. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis revealed that *S. alfredii* stimulated the activities of *Flavobacteria* and *Bradyrhizobiaceae*. The association of *S. alfredii* with carbendazim-degrading bacterial strains enhanced the degradation of carbendazim by changing microbial activity and community structure in the soil. The results demonstrated that association of *S. alfredii* with carbendazim-degrading bacterial strains is promising for remediation of Cd and carbendazim co-contaminated soil.

Keywords Bioremediation · Carbendazim-degrading bacteria · Cd · Co-contamination · *Sedum alfredii* · Biodegradation

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Introduction

In many parts of the world, agricultural soil is slightly or moderately contaminated by cadmium (Cd) (Li et al. 2010a). Cd is a heavy metal of great concern in agricultural ecosystems because of its high toxicity to animals and human health. Toxicity of Cd results in severe damage to kidneys and lungs and other pathological symptoms such as itai-itai disease through ingestion and/or inhalation (Nogawa et al. 1983). Carbendazim is a systemic broadspectrum fungicide controlling fungal pathogens on arable crops, fruits, vegetables, and herbs. It is the most widely used fungicide in China (Li et al. 2011). Repeated applications of carbendazim may result in a high residual in soil (Burrows and Edwards 2004). The residue of carbendazim can be taken up by crops and transferred along the food chain to humans. Toxic effects of down-regulation of humoral immunity (Singhal et al. 2003) and spermatogenic

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failure (Yu et al. 2009) have been reported after carbendazim exposure. Cd and carbendazim are typical pollutants in vegetable fields in the suburbs of many cities in China (Li et al. 2011). Although it is difficult to find a real soil with both high Cd and carbendazim concentrations, co-contamination of Cd and carbendazim often occurs in vegetable fields due to the increased application of sewage sludge and wastewater irrigation (Li et al. 2010a), as well as pesticides, fungicides, and chemical fertilizers (Thawornchaisit and Polprasert 2009).

Phytoremediation is considered as a cost-effective and environmentally friendly approach to remediate heavy metal contaminated soil using hyperaccumulators or accumulators (Salt et al. 1998). *Sedum alfredii* is a Cd-hyperaccumulator native to China (Yang et al. 2004). The wild population of *S. alfredii* has a strong ability to take up and accumulate Cd, and the concentrations of Cd in leaves and stems can reach a maximum of 9,000 and 6,500 mg kg^{-1}, respectively (Yang et al. 2004). Bioaugmentation can be used for the removal of pesticides and fungicides (Pattanasupong et al. 2004). Recently, some studies have been conducted on remediation of carbendazim by degrading bacterial strains (Fang et al. 2010; Wang et al. 2010; Xu et al. 2007). Terrestrial ecosystems are more often co-contaminated, but no studies have been conducted on the remediation of Cd and carbendazim co-contaminated soil. The key element for successful bioremediation of heavy metal and organic chemical co-contaminated soil is to choose a suitable plant–microbe partnership (Yu et al. 2011). The partnerships between plants and microbes may include: (1) plant can provide a large amount of root biomass and carbon to support microbial activity in soil (Huang et al. 2004); (2) microorganisms can stimulate plant growth and protect the plant from metal toxicity (Yu et al. 2011). Therefore, it is hypothesized that a Cd-hyperaccumulator plant associated with carbendazim-degrading microbial strains may be a potential combination for remediating Cd and carbendazim co-contaminated soils.

A pot experiment was conducted to evaluate if under extremely condition that the soil was severely contaminated by both Cd and carbendazim, it is possible or not to simultaneously clean the two contaminants using *S. alfredii* and carbendazim-degrading bacterial strains. The objectives of this study were thus: (1) to investigate the potential of *S. alfredii* associated with carbendazim-degrading bacterial strains (*Bacillus subtilis*, *Paracoccus* sp., *Flavobacterium*, and *Pseudomonas* sp.) for remediation of Cd and carbendazim co-contaminated soil; (2) to examine the relationship between the changes of soil microbial activity and community structure and carbendazim degradation; and (3) to explore the mechanisms of plant–microbe interactions during the bioremediation process. Results obtained from this study are expected to provide some insights into the feasibility of utilizing plant–microbe partnerships for the remediation of Cd and carbendazim co-contaminated soils.

Materials and methods

Plant culture and inoculum preparation

Hyperaccumulator *S. alfredii* was collected from an old Pb/Zn mined area of Zhejiang province, China. Prior to pot experiment, the plants were cultured for 4 weeks in hydroponics (Yang et al. 2004). Carbendazim-degrading strains were provided by the Institute of Pesticide and Environmental Toxicology of Zhejiang University, as mixed cultures of four bacterial strains (*B. subtilis*, *Paracoccus* sp., *Flavobacterium*, and *Pseudomonas* sp.).

Soil and pre-incubation

Soil was collected from the 0–15 cm surface layer on the farm of Zhejiang University, Hangzhou, China, which was carbendazim free but moderately contaminated with Cd (1.1 mg kg^{-1}). After removal of visible pieces of plant materials, grit, earthworms, etc., the soil samples were air-dried, ground, and passed through a 2-mm sieve prior to use. The soil had 1.77 g kg^{-1} total N, 4.15 g kg^{-1} total P, 1.58 g kg^{-1} total K, 19.87 g kg^{-1} organic matter content (OM), 14.66 $cmol_c$ kg^{-1} CEC, pH 5.3 and 1.1 mg kg^{-1} total Cd.

Two levels of contaminants were established in the pre-treated soil: a low level (1.1 mg kg^{-1} Cd and 40.0 mg kg^{-1} carbendazim), and a high level (6.0 mg kg^{-1} Cd and 140.0 mg kg^{-1} carbendazim). Cadmium was added as Cd $(NO_3)_2$ dissolved in deionized water and the Cd-spiked soils were aged for 2 months at a moisture content of 60 % of water holding capacity before carbendazim was added. The co-contaminated soils were then pre-incubated for 2 weeks prior to use for pot experiments. At the end of pre-incubation, the concentrations of Cd and carbendazim at the low contaminant level were 1.1 and 20.5 mg kg^{-1}, respectively, and the corresponding values were 5.8 and 116.9 mg kg^{-1} for the high contaminant level, respectively.

Pot experiment

Four treatments for each of the two contaminant levels were established: (1) low level control soil (L-CK), (2) soil inoculated with carbendazim-degrading strains (L-C), (3) soil planted with *S. alfredii* (L-*S*) and (4) *S. alfredii* associated with carbendazim-degrading strains (L-*S*+C); the same treatments were also applied to the high contaminant level soils, i.e. H-CK, H-C, H-*S*, and H-*S*+C. Each treatment had six replicates. For the treatments of C and *S*+C, bacterial suspension (15 ml, 10^{10} cfu ml^{-1}) was spiked to each pot containing 1 kg soil, then homogenized. For the treatments of *S* and *S*+C, six pre-cultured *S. alfredii* with a similar size (6–8 cm high) were transplanted to each pot. All pots were placed in green house under controlled conditions of 16 h light, at 30°C and

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8 h dark, at 22°C, and the plants were monitored daily and watered as necessary. Pot experiments were conducted for 180 day, from May to November 2010. During the first 32 days, soil samples were collected for carbendazim analysis; at 32 days, soil was also analyzed for microbial biomass carbon, dehydrogenase activity and microbial community structure. At 180 days, *S. alfredii* was harvested, washed with deionized water, and separated into roots and shoots. The freeze-dried samples were used for analysis of dry biomass and concentrations of Cd and carbendazim. Soil was simultaneously sampled and analyzed for Cd and carbendazim.

Analytical methods

Determination of Cd

Plant samples (0.1 g) of each treatment were digested with a mix of 5 mL of HNO_3+1 mL of $HClO_4$, and the digest was transferred to a 50 ml volumetric flask, made up to volume and filtered (Shentu et al. 2008). Total Cd in the soil was analyzed by digestion with a mix of 5 mL of HNO_3+1 mL of $HClO_4$+1 mL of HF (Shentu et al. 2008). Cd concentrations of the samples were determined by ICP-MS (Agilent 7500a, USA).

Determination of carbendazim

For extraction of residual carbendazim, 5 g soil was extracted by 20 ml of methanol, shaking at 200 rpm for 2 h in a rotary shaker and ultra-sonication for 20 min. Then, the samples were centrifuged at 3,000×*g* for 10 min. The supernatants were collected and passed through 0.22-μm syringe filters for HPLC (Agilent 1200, USA) analysis. The chromatographic separation was achieved on an Eclipse XDB-C_{18} column (150×4.6 mm i.d., 5 μm) with elution of methanol/water (65:35, *v*/*v*) at a flow rate of 0.8 mL min^{-1}. Carbendazim was detected using a UV–Vis detector at 281 nm, and the retention time was 2.78 min. The concentration of carbendazim in *S. alfredii* was analyzed following the method described by Su et al. (2003). Experiments on carbendazim recovery were carried out by adding a known concentration of carbendazim standards (10 mg kg^{-1}) to uncontaminated soil and plant. The results showed a recovery of 92.5±2.9 % and 90.1±4.7 % for soils and plants, respectively. Limit of detection for carbendazim was 0.1 mg kg^{-1}.

Soil microbial biomass carbon and dehydrogenase activity analysis

Microbial biomass carbon was determined using the fumigation–extraction method (Vance et al. 1987) and dissolved carbon in the K_2SO_4 extracts was measured using a TOC Analyzer (multi N/C 3100). Soil dehydrogenase activity was measured by the reduction of triphenyl tetrazolium chloride to triphenyl formazan (Namkoong et al. 2002).

Microbial community analysis

Total nucleic acids were extracted using an Ultra High Purity DNA Isolation Kit for Soil (MoBio Laboratories, Solana Beach, CA). The V3 region of 16S rDNA was amplified by PCR using the primer set 357F-GC/518R (Muyzer et al. 1993). A touch down temperature controlled program was used (Muyzer et al. 1993).

Approximately 400 ng of purified PCR product was loaded onto a 8 % (*w*/*v*) polyacrylamide gel, with denaturing gradients ranging from 35 to 65 % (100 % denaturant contains 7 M urea and 40 % formamide). DGGE was conducted in 1× TAE buffer (pH 8.0) at 60°C for 4.5 h at a constant voltage of 200 V. After electrophoresis, the gels were stained with SYBR GREEN I (Sigma, USA) for 30 min following the manufacturer's instructions. Digitized DGGE images were analyzed with Quantity One image analysis software (Version 4.62, Bio-Rad, USA). Cluster analysis of DGGE band patterns was carried out using neighbor-joining cluster method.

The specific bands in the DGGE gel were determined by excision of the bands, elution in sterilized PCR water, re-amplification with the primer set 357F/518R, and cloning of the amplicons into the *pEASY*-T1 cloning Kit (Transgen, China). Clones containing recombinant vectors were randomly selected for the presence of the appropriate 233 bp fragment by using the 357F/518R primers, and sequenced by the Invitrogen Corporation (USA). DNA sequences were compared by BLAST search program (Altschul et al. 1997) at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST). Sequences were aligned with the CLUSTAL *X*2 program and the resulting alignments were optimized by using the MEGA4.1 to construct phylogenetic trees. The neighbor-joining method was used to generate optimal tree topologies, confirmed by 1,000-fold bootstrapping.

Statistical analysis

Means of significant difference were separated at $P<0.05$ by the least significant difference test. Pearson correlation coefficients were calculated to determine the relationship between soil microbial biomass, dehydrogenase activity, microbial diversity and soil carbendazim removal efficiency. All statistical analyses were performed using SPSS 18.0 for Windows (CoHort Software, Berkeley, CA, USA).

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Nucleotide accession numbers

The 9 nucleotide sequences identified in this study were deposited in the GenBank database under accession numbers JQ027703 to JQ027711.

Results

Plant biomass

Plant growth was promoted by carbendazim-degrading strains under two contaminant levels (Table 1). Inoculation with carbendazim-degrading strains increased root and shoot weights by 22.2 % and 24.6 %, respectively, at the low contaminant level, and the corresponding values for the high contaminant level were 35.3 % and 24.6 %, respectively.

Cd phytoextraction efficiency

The concentrations of Cd in roots and shoots of *S. alfredii* were 20.3–23.0 mg kg^{-1} and 109.0–124.9 mg kg^{-1} respectively at the low contaminant level, and 47.0–63.2 mg kg^{-1} and 151.7–180.4 mg kg^{-1} respectively at the high contaminant level (Table 1). Bacterial inoculation decreased Cd concentrations in shoots of *S. alfredii* by 12.8 % at the low and 15.9 % at the high contaminant level.

Shoot Cd accumulation ($M_{stem} \times W_{stem} + M_{leaf} \times W_{leaf}$; M Cd concentration; W dry biomass) in *S. alfredii* reached 354.8.2–385.8 μg pot^{-1} and 454.5–475.8 μg pot^{-1} at the low and the high contaminant level, respectively (Table 1). The bacterial inoculation resulted in an increase in Cd accumulation by 8.76 % and 4.69 %, respectively for the low and high contaminant level.

The phytoextraction efficiency is defined as the ratio of metal accumulation in shoots to that in soil (Mertens et al. 2005), which is calculated as follows:

$$\text{Phytoextraction efficiency}(\%) = \frac{M_{shoot} \times W_{shoot}}{M_{soil} \times W_{soil}} \times 100\%$$

where M_{shoot} is Cd concentration in shoots (in milligrams per kilogram); W_{shoot} is the shoot dry weight (in grams); M_{soil} is the Cd concentration in soil (in milligrams per kilogram) and W_{soil} is the amount of soil in a pot (g). As shown in Table 1, in the treatments of *S. alfredii* (*S* and *S*+C), Cd phytoextraction efficiencies were 32.3–35.1 % at the low level, and inoculation with carbendazim-degrading strains significantly ($P < 0.05$) increased Cd phytoextraction efficiencies. Cd phytoextraction efficiencies were 7.84–8.20 % at the high contaminant level, but no significant differences were found between *S* and *S*+C.

Table 1 Influence of carbendazim-degrading bacterial strains on dry biomass, Cd concentration and Cd accumulation of *S. alfredii*, and Cd phytoextraction efficiency

Treatments[a]		Dry biomass (g pot^{-1})		Cd concentration (mg kg^{-1} DW)		Cd accumulation[b] (μg pot^{-1})		Cd phytoextraction efficiency (%)
		Root	Shoot	Root	Shoot	Root	Shoot	
Low contaminant level	*S*	0.21±0.01	2.84±0.10	23.04±0.50*	124.9±0.14**	4.84±0.24	354.8±11.51	32.25±1.16
	S+C	0.27±0.01*	3.54±0.08*	20.27±0.78	109.0±1.35	5.47±0.38*	385.8±12.12*	35.07±1.20*
High contaminant level	*S*	0.17±0.01	2.52±0.05	63.22±2.15**	180.4±1.17*	10.75±0.52	454.5±13.65	7.84±0.77
	S+C	0.23±0.00*	3.14±0.02*	47.03±0.80	151.7±4.62	10.82±0.42	475.8±16.38	8.20±0.86

*$P<0.05$ significant difference between *S* and *S*+*C* treatments at each contaminant level within the same columns

**$P<0.01$ significant difference between *S* and *S*+*C* treatments at each contaminant level within the same columns

Data are means ± SD of six replicates ($n=6$)

[a] Low contaminant level: Cd and carbendazim were 1.1 and 20.5 mg kg^{-1}; high contaminant level: Cd and carbendazim were 5.8 and 116.9 mg kg^{-1}; *S* cultivation with *S. alfredii*; *S*+*C S. alfredii* associated with carbendazim-degrading bacterial strains

[b] Shoot Cd accumulation

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Carbendazim removal from soil

Carbendazim removal in soil was enhanced by planting of *S. alfredii* and/or inoculation of carbendazim-degrading strains during the first 32 days (Fig. 1). At the low contaminant level, during the first 14 d, the fastest removal occurred for the *S* treatment, with 44.7 % of carbendazim being removed. However, at 32 d, a significant ($P<0.05$) increment of carbendazim removal was observed in *S*+C treatment, which reached 83.3 %. This value was significantly higher than that obtained from the microcosms inoculated with carbendazim-degrading strains alone (68.6 %) or planted with *S. alfredii* alone (65.2 %), indicating that *S. alfredii*–carbendazim-degrading strains interaction enhanced the removal of carbendazim at the low contaminant level. At high contaminant level, during the first 14 days, the fastest removal occurred for the C treatment (12.4 %); however, after 14 d, carbendazim was degraded slowly in C. The removal efficiency of carbendazim at 32 days was in the order of *S*+C (60.8 %)>*S* (56.9 %)>C (24.7 %)> CK (17.3 %), and the difference between *S*+C and *S* treatments was not significant, indicating that *S. alfredii* was more effective than carbendazim-degrading strains in enhancing carbendazim degradation at the high contaminant level.

The concentrations of carbendazim in soil were lower than 1 mg kg^{-1} when the experiment was finished (Table 2). The residual percentages were 0.83, 0.39, 0.24, and 0.20 %, respectively for CK, C, *S*, and *S*+C treatments, at the low contaminant level. The corresponding values were 0.50, 0.23, 0.19 and 0.14 %, respectively at the high contaminant level.

Soil microbiological characteristics

Soil microbial biomass C and dehydrogenase activities

Microbial biomass carbon is a good indicator of soil quality (Pascual et al. 1997). It was increased by 46.3–67.9 % and 49.43–71.49 % by the growth of *S. alfredii* and carbendazim-degrading strains, respectively, as compared to the control, under two contaminant levels (Fig. 2). Dehydrogenase activity assays have often been used to assess the biological activity of microbial populations in soil, i.e., as an index of total microbial activity (Maliszewska-Kordybach and Smreczak 2003). In this study, dehydrogenase activities were increased by 88.8–158.4 % and 64.21–69.67 %, respectively by the growth of *S. alfredii* and carbendazim-degrading strains, under two contaminant levels (Fig. 2).

Soil microbial community

The band patterns in the treated and control soils were different, suggesting that planting of *S. alfredii* and/or inoculation of carbendazim-degrading strains changed the bacterial communities (Fig. 3). The presence of *S. alfredii* (L-*S*, L-*S*+C and H-*S*, H-*S*+C) appeared to stimulate the bands phylogenetically assigned to *Flavobacteria* (band no. 2) and *Bradyrhizobiaceae* (band no. 5), as compared with the unplanted treatments. Band No. 3 showed 100 % sequence similarity to *Pseudomonas* sp. CBW, likely the *Pseudomonas* strain inoculated into the soil, indicating that this bacterium survived and had become the dominant species in the soil, as evidenced by the strengthened band No. 3 in the treatments with carbendazim-degrading strains inoculation (L-C, L-*S*+C and H-C).

Shannon index were used to interpret the diversity of bacterial community. Cultivation of *S. alfredii* and/or inoculation of carbendazim-degrading strains increased soil microbial diversity (Fig. 3). Cluster analysis of the DGGE patterns demonstrated that the bacterial community profiles were clustered into four groups (Fig. 3), i.e. Group 1, H-*S*+C; Group 2, L-*S* and L-CK; Group 3, H-*S* and H-CK; and Group 4, L-C, H-C, and L-*S*+C. As shown in neighbor-joining phylogenetic tree (Fig. 4), the dominant bands in the bacterial community were mainly assigned to *Actinobacteria* (*Rhodococcus* and *Rubrobacterales*), *Alphaproteobacteria* (*Bradyrhizobiaceae*,

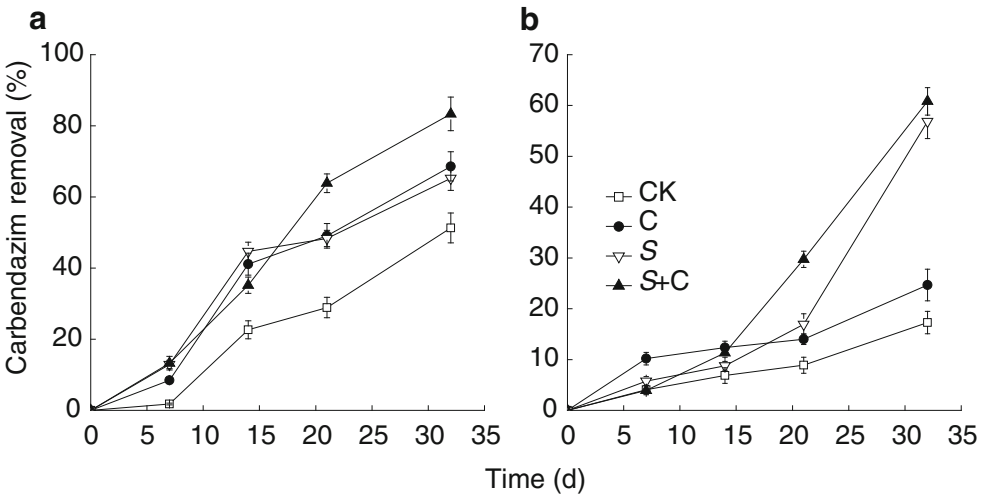

Fig. 1 Carbendazim removal in soil during the first 32 days of experiment. **a** Low contaminant level: carbendazim was 20.5 mg kg^{-1}. **b** High contaminant level: carbendazim was 116.9 mg kg^{-1}. *CK* control soil; *C* inoculation with carbendazim-degrading bacterial strains; *S* cultivation with *S. alfredii*; *S+C S. alfredii* associated with carbendazim-degrading bacterial strains

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Table 2 Soil Cd and carbendazim concentrations after remediation

Treatments[a]		Cd concentration (mg kg^{-1} DW)		Carbendazim (mg kg^{-1} DW)
		Total Cd	Mehlich 3-Extractable Cd	
Low contaminant level	CK	0.99±0.01a	0.22±0.01a	0.17±0.01a
	C	0.96±0.01b	0.22±0.01a	0.08±0.01b
	S	0.61±0.05c	0.14±0.01b	0.05±0.01c
	S+C	0.56±0.04c	0.13±0.01b	0.04±0.00c
High contaminant level	CK	5.62±0.03a	4.33±0.06a	0.58±0.03a
	C	5.48±0.05b	4.30±0.03a	0.27±0.01b
	S	5.10±0.10c	4.01±0.07b	0.22±0.01c
	S+C	4.91±0.05d	3.94±0.04b	0.16±0.01d

Data are means ± SD of six replicates (n=6). Different letters (a–d) indicated significant (P<0.05) differences among all treatments at each contaminant level within the same columns.

[a] Low contaminant level: Cd and carbendazim were 1.1 and 20.5 mg kg^{-1}; High contaminant level: Cd and carbendazim were 5.8 and 116.9 mg kg^{-1}; *S* cultivation with *S. alfredii*; *S*+C *S. alfredii* associated with carbendazim-degrading bacterial strains.

Rhodobacter and *Sphingomonas*), *Gammaproteobacteria* (*Pseudomonas* and *Luteibacter*), *Flavobacteria* and *Chloroflexi* or *Planctomycetes* (91 % and 90 % of sequence similarity to *Chloroflexi* and *Planctomycetes*, respectively).

Discussion

Soil Cd removal by integrated bioremediation system

In this study, the Cd concentrations in shoots of *S. alfredii* exceeded the threshold value (100 mg kg^{-1}) for a Cd-hyperaccumulator (Salt et al. 1998), and followed the order of shoots > roots. This result did concur with previous study reported by Yang et al. (2004), which confirmed that *S. alfredii* has an extraordinary ability to take up Cd and transfer it to the shoots.

Inoculation with carbendazim-degrading strains increased biomass of the hyperaccumulator *S. alfredii*, as compared with uninoculated treatments (Table 1). Associative and free-living microorganisms may contribute to the growth of plants through a variety of mechanisms including direct effects on nutrient availability and enhancement of root growth (Raaijmakers et al. 2009). Yu et al. (2011) have also found that PAH-degrading bacteria stimulate the growth of ryegrass. On the other hand, the concentrations of Cd in *S. alfredii* were significantly (P<0.01) decreased with bacterial inoculation. Chanmugathas and Bollag (1987) have reported that, in a severely disturbed ecosystem, soil microorganisms contribute to plant establishment by immobilizing heavy metals in

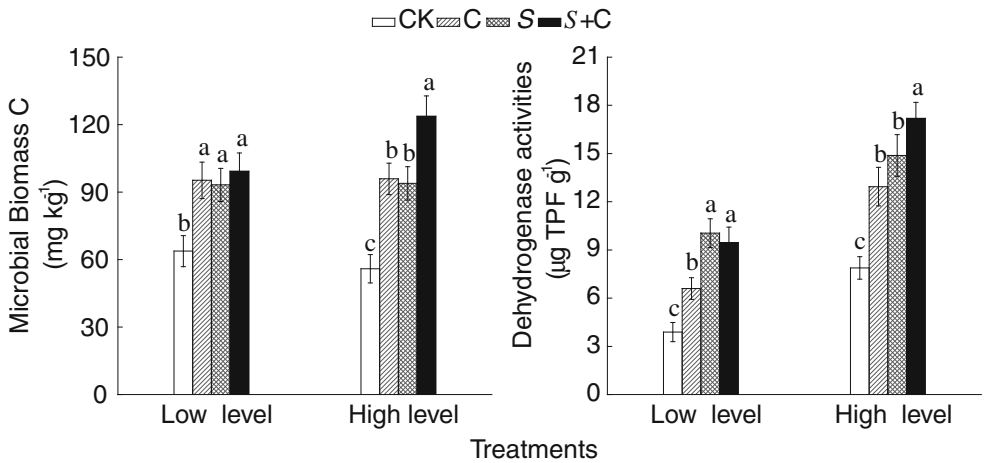

Fig. 2 Microbial biomass carbon and dehydrogenase activity in soil influenced by Cd, carbendazim, *S. alfredii* and carbendazim-degrading bacterial strains. Columns denoted by different letters (**a**–**c**) indicate significant (p<0.05) differences among the treatments. Low level (Cd and carbendazim were 1.1 and 20.5 mg kg^{-1}, respectively); High level (Cd and carbendazim were 5.8 and 116.9 mg kg^{-1}, respectively). *CK* control soil; *C* inoculation with carbendazim-degrading bacterial strains; *S* cultivation with *S. alfredii*; *S*+*C S. alfredii* associated with carbendazim-degrading bacterial strains

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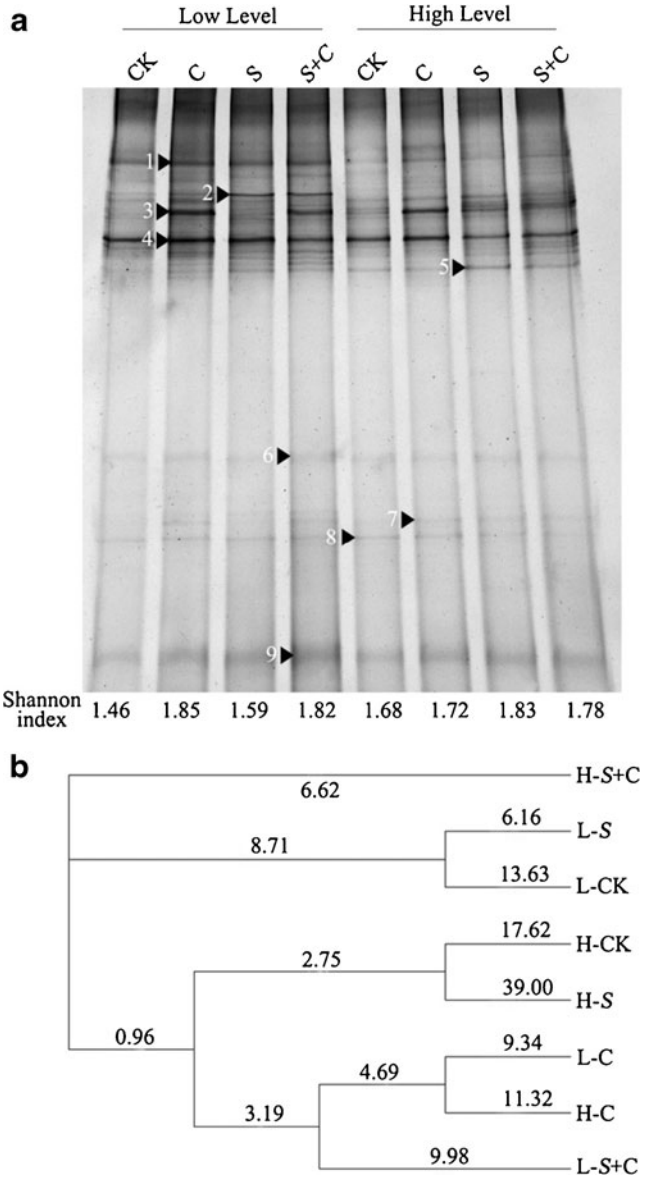

Fig. 3 Composition of the microbial community in soil. **a** DGGE profiles of the soil bacterial 16S rRNA fragments amplified with the primer set 357F-GC/518R. **b** Cluster analysis of DGGE band patterns. L refers to low contaminant level (Cd and carbendazim were 1.1 and 20.5 mg kg^{-1}, respectively); H refers to high contaminant level (Cd and carbendazim were 5.8 and 116.9 mg kg^{-1}, respectively). *CK* control soil; *C* inoculation with carbendazim-degrading bacterial strains; *S* cultivation with *S. alfredii*; *S+C S. alfredii* associated with carbendazim-degrading bacterial strains

rhizospheric soil and reducing heavy metal concentrations in plant. The bacterial inoculation increased Cd accumulation in the harvestable aboveground biomass and subsequently the phytoextraction efficiency, because the increase of *S. alfredii* biomass was high enough to compensate for the decrease in Cd concentrations of plant tissues. Huang et al. (2004) have also reported that addition of plant growth-promoting rhizobacteria does increase the pollutant removal probably by stimulating the plants to grow faster and accumulate more root biomass in heavily contaminated soils.

As shown in Table 2, Cd removal efficiencies were increased by 34.5–36.4 % in *S. alfredii* treatments (*S* and *S*+C) at the low contaminant level, as compared with unplanted treatments. The corresponding values were 9.0–9.8 % for the high contaminant level. Most of the Cd removal from the soil is due to *S. alfredii* uptake. Mehlich 3-extractable Cd is considered a good index for estimating bioavailability of soil Cd in numerous soils around the world (Mehlich 1984). Bacterial inoculation had no significant influence on Mehlich 3-extractable concentration of Cd in soil. On the other hand, Mehlich 3-extractable Cd was significantly lower in the *S. alfredii* treatments (*S* and *S*+C), as compared with unplanted treatments, regardless of contaminant level (Table 2). The decrease in Mehlich 3-extractable concentration of Cd in the soil after the growth of *S. alfredii* was observed to account for only a small part of the Cd accumulation in *S. alfredii* (Table 2), indicating that part of the Cd uptake by *S. alfredii* may have come from less available Cd pools in soil. This result concurred with the finding of Li et al. (2010b), who reported that organic acids excreted by rhizospheric bacteria of *S. alfredii* could form soluble complexes with Cd and thus promote Cd availability for this plant.

Soil carbendazim removal by integrated bioremediation system

Carbendazim-degrading strains have been demonstrated to enhance the dissipation of carbendazim from soil (Fang et al. 2010; Kalwasinska et al. 2008; Wang et al. 2010). In this study, carbendazim-degrading strains inoculated into the soil were *B. subtilis*, *Paracoccus* sp., *Flavobacterium*, and *Pseudomonas* sp., but *B. subtilis* and *Paracoccus* sp. were not detected in soil by DGGE analysis (Figs. 3 and 4). These results indicate that *B. subtilis* and *Paracoccus* sp. might not survive in soil, due to competition from indigenous microbes or nutrition deficiency (Wenzel 2009). Among the carbendazim-degrading strains, *Pseudomonas* sp. seemed to play an important role in carbendazim degradation.

This study showed growth of *S. alfredii* significantly increased carbendazim degradation in soil. Obviously, the enhanced dissipation of carbendazim in planted versus unplanted soil would overwhelmingly derive from *S. alfredii* direct uptake and promoted biodegradation. However, the amount of carbendazim directly accumulated in *S. alfredii* only accounted for approximately 0.05 % and 0.02 % of the dissipated carbendazim, respectively, for the low and high contaminant soil (data not shown). Therefore, the enhanced dissipation of carbendazim would derive from promoted biodegradation, but not by plant direct uptake. These results did concur with the findings of Yu et al. (2011) and Teng et al. (2011), who conclude that enhanced dissipation of PAHs by

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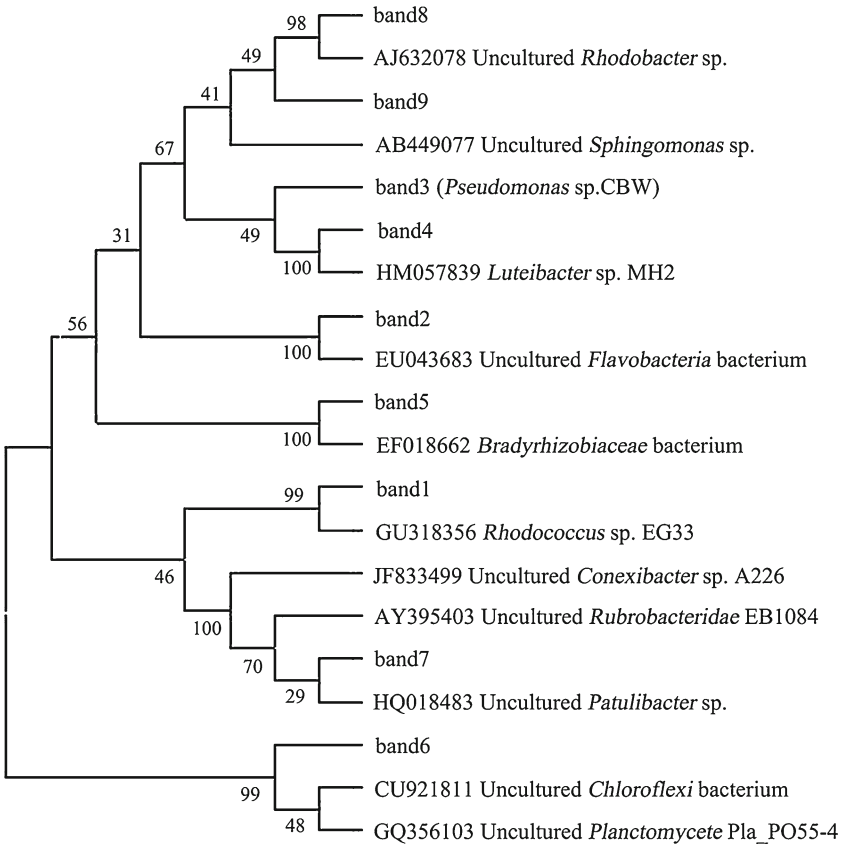

Fig. 4 Phylogenetic analysis showing relationships of clones sequenced from 16S rRNA gene amplified with the primer set 357F-GC/518R. Bootstrap support values with 1,000 replicates are given along the branches

mycorrhizal ryegrass or alfalfa does derive from plant promoted biodegradation, but not by plant uptake.

At the low contaminant level, carbendazim-degrading strains significantly ($P<0.05$) enhanced carbendazim degradation, and the highest degradation rate of carbendazim (83.3 %) was observed in *S*+C treatment at the first 32 days (Fig. 1), indicating greater potential of carbendazim-degrading strains associated with *S. alfredii* in removing soil carbendazim. However, at the high contaminant level, *S. alfredii* was more effective than carbendazim-degrading strains in removing carbendazim (Fig. 1), because the growth of carbendazim-degrading strains were inhibited by high concentration of carbendazim in the soil, as evidenced by the fact that the band of *Pseudomonas* sp. (one of carbendazim-degrading strains) was brighter at the low than the high contaminant level (Fig. 3). Fang et al. (2010) have also reported that, the growth of *Pseudomonas* sp. is inhibited by 100 mg L^{-1} of carbendazim in mineral salt medium. On the other hand, *S. alfredii*, as a Zn/Cd-hyperaccumulator, had an extraordinary ability to tolerate a high level of pollutants, hence grew well in soil polluted by high concentration of carbendazim. Furthermore, *S. alfredii* increased soil microbial activities (Fig. 2), enriched microbial diversities and changed its community structure (Fig. 3). Long et al. (2009) have also reported a stimulatory effect of *S. alfredii* on soil microbial growth, activities and metabolic profiles. It is supposed that the changes of soil microbes may contribute to the degradation of carbendazim. This hypothesis was confirmed by Pearson correlation analysis: the Pearson correlation coefficients between carbendazim removal and soil microbial biomass carbon, dehydrogenase activity and microbial diversity were 0.742, 0.884, and 0.836, respectively at the low contaminant level, and 0.785, 0.898, and 0.918, respectively at the high contaminant level. Margesin et al. (2000) have also reported that hydrocarbon removal is positively correlated with dehydrogenase activity. *Flavobacteria* and *Bradyrhizobiaceae* were stimulated in the treatments with *S. alfredii* (*S* and *S*+C), as compared to those without plants (Fig. 3). These two stimulated strains might actively participate in carbendazim removal. It was consistent with Joner and Leyval (2003), who have noted that clover and ryegrass stimulate PAH degradation by modifying microbial community. Cea et al. (2010) have noted that microbial communities within a contaminated ecosystem tend to be dominated by those organisms capable of utilizing or surviving toxic contaminants, and the most sensitive microbial species are often replaced by more tolerant ones in these sites. From the above results, it seems reasonable to conclude that the association of *S. alfredii* with carbendazim-degrading strains contributed to the biodegradation of carbendazim by promoting microbial activity, enriching microbial diversity and modifying microbial community structure. Various studies have already shown the importance of microbial population on effective bioremediation of xenobiotics, i.e., ryegrass does enhance

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dissipation of PAH by increasing microbial activity (Binet et al. 2000), enriching microbial functional diversity (Kirk et al. 2005), and altering the microbial community structure in rhizosphere (Kirk et al. 2005).

This study showed carbendazim removal from contaminated soil by biaugmentation with carbendazim-degrading strains, and also by the soil indigenous microorganisms. DGGE analysis revealed the presence of *Rhodococcus* sp., *Pseudomonas* sp., and *Sphingomonas* sp. in soil during carbendazim remediation (Fig. 4). This result confirmed previous findings that these strains are capable of degrading carbendazim at relatively low level (Fang et al. 2010; Kalwasinska et al. 2008; Xu et al. 2007). Other dominant members in the soil (*Patulibacter*, *Conexibacter*, *Bradyrhizobiaceae*, *Rhodobacter*, *Luteibacter*, *Flavobacterium* and *Chloroflexi* or *Planctomycetes*) might also actively participate in carbendazim removal. Further studies are needed to evaluate the functions of these strains in carbendazim removal.

Conclusion

The goal of this study was to investigate the potential of *S. alfredii* associated with carbendazim-degrading strains for remediation of Cd and carbendazim co-contaminated soil. Carbendazim-degrading strains improved Cd removal in soil by increasing biomass of *S. alfredii*, and *S. alfredii* associated with carbendazim-degrading strains enhanced the degradation of carbendazim by promoting microbial activities. This study also showed soil microbial activity and community structure changed during the remediation. The results demonstrated that, *S. alfredii* combined with carbendazim-degrading strains is a suitable plant–microbe partnership for the remediation of Cd and carbendazim co-contaminated soil. Further studies are needed to elucidate the metabolic pathway of carbendazim degradation in plant–microbe associations and the molecular mechanisms that lead to carbendazim degradation and transformations.

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