Microbial remediation of a pentachloronitrobenzene-contaminated soil under *Panax notoginseng*: A field experiment

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ABSTRACT

Pentachloronitrobenzene (PCNB) is an organochlorine fungicide that is mainly used in the prevention and control of diseases in crop seedlings. Microbial remediation was explored to assess the potential role of a PCNB-degrading bacterial isolate, *Cupriavidus sp.* YNS-85, in the remediation of a PCNB-contaminated soil on which *Panax notoginseng* was grown. The following three treatments were used: i) control soil amended with wheat bran but without YNS-85, ii) soil with 0.15 kg m⁻² of solid bacterial inoculum (A), and iii) soil with 0.30 kg m⁻² of solid bacterial inoculum (B). The removal of soil PCNB during the microbial remediation was monitored using gas chromatography. Soil catalase and fluorescein diacetate (FDA) esterase activities were determined using spectrophotometry. In addition, cultivable bacteria, fungi, and actinomycetes were counted by plating serial dilutions, and the microbial biodiversity of the soil was analyzed using BIOLOG. After 1 year of *in situ* remediation, the soil PCNB concentrations decreased significantly by 50.3% and 74.2% in treatments A and B, respectively, when compared with the un inoculated control. The soil catalase activity decreased in the presence of the bacterial isolate, the FDA esterase activity decreased in treatment A, but increased in treatment B. No significant changes in plant biomass, diversity of the soil microbial community, or physiochemical properties of the soil were observed between the control and inoculated groups (*P* < 0.05). The results indicate that *Cupriavidus sp.* YNS-85 is a potential candidate for the remediation of PCNB-contaminated soils under *P. notoginseng*.

Key Words: *Cupriavidus sp.* YNS-85, enzyme activities, *in-situ* remediation, PCNB removal, soil

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INTRODUCTION

Pentachloronitrobenzene (PCNB) is an organochlorine fungicide that is mainly used in the prevention and control of diseases in crop seedlings. Pentachloronitrobenzene is persistent in soils (half-life 5–10 months) and can be biologically accumulated and magnified through the food chain, resulting in a significant risk to human health due to its carcinogenic, teratogenic, and mutagenic potential (Shin et al., 2003; Wen et al., 2010; Tas and Pavlostathis, 2014). The value of the lethal concentration (LC50) of PCNB is 0.55 mg L⁻¹ in rainbow trout and 0.1 mg L⁻¹ in crapat arlequin (U.S. National Library of Medicine, 1995). Extensive use has made PCNB contamination a global environmental problem (Wang et al., 2015). Pentachloronitrobenzene has been used extensively as a soil fungicide, especially for the prevention of soil-borne diseases in Chinese farmlands where Chinese notoginseng (*Panax notoginseng*) is grown (Li et al., 2009). Chinese notoginseng is produced mainly in Wenshan, Yunnan Province, southern China, and it is traditionally valued for its protective action against cerebral ischemia, beneficial effects on the cardiovascular system, and hemostatic action in traditional Chinese medicine (Ng, 2006). Although the use of PCNB is prohibited in many countries, its biological accumulation, persistence, and low biodegradability have led to substantial accumulation of PCNB in plants, soils and water in some ecosystems (Arora et al., 2012). In China, quality and technical supervision rules for the ginseng product-quality standard have set the limit for ginseng PCNB as 0.1 mg kg⁻¹. A previous study detected PCNB concentrations in *P. notoginseng* of up to hundreds of µg kg⁻¹ (Leung et al., 2005). Therefore, there is considerable interest in the control or prevention of pesticide pollution in soils used to grow these medicinal plants.

Bioremediation is an emerging strategy in which plants and bacteria are used for the rehabilitation of polluted sites. Although bioaccumulation of PCNB has been noted in alfalfa (Li and Yang, 2013), phytoremediation is often...
regarded as slow and incomplete, and it may be impeded by the low bioavailability of PCNB (Teng et al., 2015). In addition, the environmental fate, and even the bioremediation process, is influenced by certain abiotic and biotic effects (Gorontzy et al., 1994). Any bioaugmentation strategies require a deep understanding of the ecology of contaminant degradation because each contaminated site is unique with regard to potential hazards for ecosystems and human health (Vogt and Richnow, 2013). The introduction of indigenous microorganisms in bioremediation might address some of the weaknesses of this technique. The diverse metabolic capacities of microbes make them valuable tools for the restoration of contaminated sites. However, the microbial transformation of PCNB is mainly focused on fungi (e.g., Fusarium spp., Sporothrix cyanescens, Mucor racemosus, and white rot fungi) and actinomycetes (e.g., Streptomyces aureofaciens) (Lièvremont et al., 1998; Arora et al., 2012). Only a few bacterial species, including a Labrys strain, capable of PCNB degradation, have been found (Li et al., 2011), and the degradation capacity of both fungal and bacterial strains has been confirmed in only laboratory studies (Liu et al., 2011; Li and Yang, 2013). Little information is available about whether they would be effective in PCNB degradation under field conditions. Soil enzyme activities and microbial biodiversity have also not been explored during the biodegradation of PCNB in the field. It is necessary to select suitable candidate organisms for the removal of PCNB (Lièvremont et al., 1998), particularly in field studies because laboratory conditions cannot simulate the more complicated conditions in the field.

A PCNB-degrading bacterium, Cupriavidus sp. YNS-85, was isolated in our laboratory. The main goal of the present study was to determine whether this isolate could degrade PCNB in soil under P. notoginseng. Moreover, the activity and biodiversity profiles of soil enzymes were assayed after 1 year of in-situ bioremediation. Soil physicochemical properties and plant biomass were also evaluated during the biodegradation of PCNB. The results of this study should contribute to our understanding of the bioremediation potential of the bacterial isolate in PCNB-contaminated fields cultivated with P. notoginseng.

MATERIALS AND METHODS

Site description and soil properties

The P. notoginseng test area is located in the southeast of Yunnan Province, South China, and our field site is located at 23°52′38″N, 104°32′40″E. Each experimental plot was 10 m² (2 m × 5 m), with a mound height of 30 cm; the study was conducted from December 2014 to December 2015.

The test soil type at the field site is a ferruginous soil according to the World Reference Base for Soil Resources system. The average PCNB concentration and physicochemical properties of the experimental soil were determined using standard methods (Li et al., 2011) and were as follows: PCNB 3.95 mg kg⁻¹, pH 6.9, total organic matter 27.3 g kg⁻¹, cation exchange capacity (CEC) 18.93 cmol kg⁻¹, total N 0.79 g kg⁻¹, total P 0.3 g kg⁻¹, total K 4.37 g kg⁻¹, hydrolyzable N 95 mg kg⁻¹, exchangeable P 0.7 mg kg⁻¹, and exchangeable K 100 mg kg⁻¹.

Experimental design and sampling

Solid bacterial inoculum was prepared by mixing a liquid suspension (optical density at 600 nm, 1.0) of Cupriavidus sp. YNS-85 with dry wheat bran at a mass ratio of 2:3. The control soil received wheat bran without the bacterial suspension. Two experimental treatments, named A and B, received 0.15 and 0.30 kg m⁻² (about 8 × 10¹¹ and 16 × 10¹¹ colony-forming units (CFU) kg⁻¹) of the solid bacterial inoculum agent, respectively. Full-grown seeds that were 6–8 cm length and 1 cm in diameter were transplanted at the test site, with each plant occupying an area of 5 cm × 6 cm. The collection of a soil sample for each treatment was replicated five times. The surface soil (5–15 cm) was collected using the five-point sampling method and mixed thoroughly. The fresh soil samples were stored at −80 °C before the test analyses. In addition, 10 plants were collected randomly from each treatment field. Whole plants containing the taproot and fibrous roots were rinsed with deionized water to remove the soil residue. After blow-drying, the fresh weight, height, and root length of the plants were determined immediately. The dry weight was then measured after oven drying at 65 °C.

Analysis of PCNB in soil

The soil samples were freeze-dried and sieved through a 0.25-mm mesh. Extraction of PCNB was performed according the methods described by Sun et al. (2007) and Wang et al. (2015). Five grams of soil was weighed into a 60-mL glass centrifugal tube with 20 mL of a methylene chloride/hexane solution (volume:volume, 1:1) and soaked overnight. The solution was ultrasonically extracted for 30 min and then centrifuged at 2 500 r min⁻¹ for 3 min. Extraction and centrifugation were repeated twice. Three samples of the collected supernatant were mixed thoroughly and then evaporated to 2 mL. The prepared solution was passed through a compound silica gel column composed of silica gel, neutral alumina, and anhydrous sodium sulfate (weight:weight:weight, 2:2:1), leached using 30 mL of a methylene chloride/hexane mixed solvent (volume:volume, 1:1), and concentrated to 1 mL with a rotary evaporator. After adding 5 mL of chromato- graphically pure n-hexane, the solution was concentrated to near dryness and then diluted to 1 mL with n-hexane for gas chromatography analysis.

In the gas chromatography analysis, the prepared sample solutions were determined using a Model 7890 gas chromato-
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The soil contents of PCNB 1 year after the bioaugmentation treatments are presented in Fig. 1. The mean PCNB contents in the control and treatments A and B after the application of solid bacterial inoculum were 2.607 ± 0.227, 2.236 ± 0.161, and 1.278 ± 0.113 mg kg⁻¹, respectively. The statistical analysis revealed that the difference in PCNB contents among the treatments was significant (P < 0.05). The treatments significantly reduced the PCNB content compared to the control. The PCNB removal percentage was calculated as (control - treatment) / control * 100%. The PCNB removal percentages for treatments A and B were 12.6% and 43.4%, respectively, while the control had a removal percentage of 0%.

**Statistical analysis**

All data in triplicate were processed using Microsoft Excel 2007 and are expressed as means ± standard deviation values. All statistical analyses were performed using the SPSS 17.0 software package. The chemical and enzymatic data were analyzed with one-way analysis of variance, and pairs of mean values were compared using Duncan’s multiple range test at 5% significance level.

**RESULTS AND DISCUSSION**

**PCNB removal**

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**RESULTS AND DISCUSSION**

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1.778 ± 0.420, and 0.682 ± 0.162 mg kg\(^{-1}\), respectively. Treatments A and B, in which 31.8% and 76.7% of PCNB was degraded, showed significantly lower PCNB contents than the control (\(P < 0.05\)). This indicates that the application of solid bacterial inoculum significantly enhanced the removal of PCNB, and with larger amount of inoculum, more PCNB was removed. The PCNB content in the control was significantly lower (\(P < 0.05\)) than the initial content in the soil (4.167 ± 0.43 mg kg\(^{-1}\)). This loss may be attributed to natural attenuation (Bento et al., 2005; Huang et al., 2007) by biodegradation, sorption, volatilization, and transformation (Gomes et al., 2013).

*Cupriavidus* sp. strain YNS-85 was isolated from a long-term PCNB-polluted field cultivated with *P. notoginseng*. This isolate has been confirmed to be able to degrade PCNB under laboratory conditions. The biodegradation efficiency was 79.4% within 7 d in solution, and 37.8% PCNB was removed from the soil 30 d after inoculation. The results of the field experiment also indicate a substantial degradation effect by the bacterium. In the laboratory experiment, metabolism of PCNB by *Cupriavidus* sp. strain YNS-85 was analyzed, and pentachloroaniline (PCA) was found to be one of the metabolites. In addition, the isolate enhanced the accumulation of its intermediate PCA, together with the removal of PCNB from spiked soil (data unpublished). However, 201 intermediates of pollutants are likely to possess higher toxicity than their parent compounds (Tas and Pavlostathis, 2014). A previous study has shown that the toxicity of PCA is lower than that of PCNB (Torres et al., 1996). The metabolism of PCNB by the *Cupriavidus* strain in the field experiment may have been similar to that in the laboratory, but this requires further study.

Microbial degradation has been proposed as a strategy to enhance the remediation of soils contaminated with organics (Juhasz and Naidu, 2000). Bacterial remediation of PCNB has also been demonstrated in other studies. *Nocardioides* sp. PD653 was found to require additional nutritional factors to multiply (Takagi et al., 2009), while *Alcaligenes* sp. PCNB-2, *Labrys portucalensis* PCNB-21, and *Arthrobacter nicotianae* DH19 used PCNB as the sole carbon and energy source, with a degradation rate of 83%–90% (Shin et al., 2003; Li et al., 2011; Wang et al., 2015). Furthermore, Li et al. (2011) found that the degradation of PCNB by *L. portucalensis* PCNB-21 in sterile soils was more rapid than that in non-sterile soils, indicating that the remediation efficiency of microbial agents might be constrained by the indigenous soil microflora. Some studies have shown that determination of ferrous ion concentration in soil can also look for microbial effects on PCNB degradation (Hakala et al., 2007). The indigenous bacteria *Klebsiella* spp. have a better effect than the non-indigenous *Pseudomonas* spp. on the repair of tributyltin (Abubakar et al., 2016), which shows the advantage of indigenous microbial remediation.

**Soil physicochemical properties and growth of *P. notoginseng***

Differences in soil physicochemical properties among the three treatments are shown in Table I. Numerous differences in soil properties were observed between the control and initial values, and this is consistent with the results of Chen and Xu (2005) and Zeng et al. (2009). The total and available N contents were slightly higher at the higher amount of the inoculum (0.30 kg m\(^{-2}\)). Previous studies have found that microbes can change the chemical properties of the soil (Yu et al., 2011). In our study, total P and available K contents were a little higher in the inoculated plots than in the control plot, and organic matter, alkali-hydrolyzable N, and available K contents increased with the higher amount of the inoculum. However, these differences are not significant.

Ten plants were collected randomly from each treatment plot to determine whether supplementation with the bacterial isolate would hinder the growth of *P. notoginseng*. The results (Table II) show that the survival rate of the plants in treatment A was higher (69%) than that in treatment B (38%), indicating that a certain amount of solid inoculum had the potential to promote the survival of *P. notoginseng*, but an excess had the opposite effect. *Cupriavidus* sp. has been demonstrated to promote plant growth (Pereira et al., 2015). However, previous studies have shown that some plant species can accumulate organic pollutants (Tu et al., 2011). The survival rate of *Cupriavidus* sp. in treatment B was lower, and the PCNB content was lower. This indicates

**TABLE I**

| Treatment\(^{a}\) | Organic matter | Hydrolyzable N | Exchangeable | CEC\(^{b}\) |
|-----------------|----------------|----------------|--------------|-----------|
|                 | g kg\(^{-1}\) dry soil | mg kg\(^{-1}\) dry soil | mg kg\(^{-1}\) dry soil | cmol kg\(^{-1}\) |
| Control         | 10.77 ± 1.0\(^{c}\) | 0.66 ± 0.04 | 0.63 ± 0.14 | 21.28 ± 1.03 | 42.63 ± 9.93 | 22.07 ± 6.16 | 226 ± 46.99 | 11.1 ± 0.86 |
| A               | 10.24 ± 0.8 | 0.55 ± 0.05 | 0.91 ± 0.13 | 21.72 ± 1.20 | 43.37 ± 5.45 | 22.67 ± 5.94 | 280 ± 50.47 | 10.8 ± 0.65 |
| B               | 12.18 ± 1.6 | 0.69 ± 0.03 | 0.70 ± 0.08 | 20.46 ± 0.19 | 49.25 ± 3.68 | 22.66 ± 2.67 | 285 ± 40.18 | 11.5 ± 0.40 |

\(^{a}\)Control = soil amended with wheat bran but without *Cupriavidus* sp. YNS-85; A = soil with 0.15 kg m\(^{-2}\) of solid bacterial inoculum of YNS-85; B = soil with 0.30 kg m\(^{-2}\) of solid bacterial inoculum of YNS-85.

\(^{b}\)Cation exchange capacity.

\(^{c}\)Means ± standard deviations (n = 3).
that the decline in PCNB can be ascribed to the application of the bacterial agent and not to uptake by *P. notoginseng*.

**Soil FDA esterase and catalase activities**

Soil enzymes that catalyze a wide range of soil biological processes have been widely considered as effective indicators of soil “function” and general microbial activities (Killham and Staddon, 2002). Soil FDA esterase and catalase activities can represent hydrolysis and oxidation-reduction levels. Soil FDA esterase and catalase activities under different treatments are shown in Fig. 2. The FDA esterase activities in treatments A and B were significantly lower than those in the control. This indicates that the application of the solid inoculum significantly decreased the activity of FDA esterase in the soil. The disparity of the effects of the solid inoculum can be observed in the soil redox level and overall microbial activity. Numerous studies on the effects of pesticides on soil enzyme activity have been conducted (Gianfreda et al., 1995; Sannino and Gianfreda, 2001). The amount of pesticide processed is the same; mean soil redox levels decline with the addition of bacterial inoculum, and overall microbial activity may decline as a result of bacterial inoculum application. Soil catalase activity shows a significant correlation with organic C content in the soil and can be used to determine soil biomass (Stepniewska et al., 2009). Although the soil catalase activity in treatment A decreased after 1 year of microbial remediation, it was not significantly different from that in the control. The soil catalase activity in treatment B was significantly lower than those in the control and treatment A. This may be due to the larger amount of bacterial inoculum negatively affecting other microorganisms; therefore, treatment A had the more appropriate application rate.

**Soil cultivable microbial counts and microbial biodiversity**

The counts of the cultivable bacteria, fungi, and actinomycetes are shown in Table III. The bacterial count decreased with an increase in the amount of solid inoculant (Control > A > B), possibly because of competition between *Cupriavidus* sp. YNS-85 and the bacterial microflora (Fontaine and Barot, 2005). The fungal counts declined in the following sequence: B > Control > A. The sequence of the actinomycete counts was A > Control > B. Fungi have been found to cause black root rot in *P. notoginseng* in China (Mao et al., 2014), and the determination of soil cultivable microbial counts can explain the impact of different quantities of the solid inoculant on the microbial counts and survival rate (Table II) associated with the fungi.

The BIOLOG method is useful for the study of the functional diversity of the microbial community (Garland and Mills, 1991). Although strain YNS-85 is an indigenous bacterium isolated from the study soil, the effects of inoculation with a large number of strain YNS-85 cells on other microorganisms require further study. The average well-color development (AWCD) can be used as an indicator of soil microbial activity in terms of functional diversity, showing...
the effects of the solid bacterial agent used (Garland and Mills, 1991). The AWCD data from the soil samples under the three treatments are shown in Fig. 3. The AWCD values of treatment A increased rapidly after 48 h and reached 0.12 within 168 h of incubation, with a final value of 1.2; the AWCD values of the control and treatment B were only 0.8 and 0.85, respectively, at the end of the incubation period ($P < 0.05$). This indicates that an intermediate dose of the bacterial isolate may enhance microbial activity, but a large amount may have an effect similar to the control. This may be attributed to the competitive effect of an adequate input of the bacterial isolate on other microorganisms (Fontaine and Barot, 2005). Overall, the impact of strain YNS-85 on microbial biodiversity was only confirmed over 1 year of remediation, and thus, it may represent a promising treatment for the remediation of PCNB-contaminated soils.

**CONCLUSIONS**

Currently, microbial removal is used as a promising method for in-situ removal of many organic pesticides and pesticide residues. The data from this field experiment showed that the soil PCNB content decreased after application of the solid inoculum of *Cupriavidus* sp. YNS-85, indicating that this isolate played an important role in the removal of PCNB in soil under *P. notoginseng*. The plant survival rate, soil enzyme activity, soil cultivable microbial counts, and microbial community structure indicate that 0.15 kg m$^{-2}$ is a more suitable application rate of the solid inoculant than 0.30 kg m$^{-2}$. This study demonstrates the potential for field application of the solid inoculant of *Cupriavidus* sp. YNS-85 for the remediation of PCNB-contaminated soils. However, the relationship between the bacterium agent and fungus, whether *P. notoginseng* can bioaccumulate PCNB, and how the degradation byproducts migrate need to be studied further under field conditions.

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