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EFFECT OF CORN PLANT ON SURVIVAL AND PHENANTHRENE DEGRADATION CAPACITY OF *PSEUDOMONAS* SP. UG14LR IN TWO SOILS

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A study was undertaken to assess if corn (Zea mays L.) can enhance phenanthrene degradation in two soils inoculated with* Pseudomonas *sp. UG14Lr. Corn increased the number of UG14Lr cells in both soils, especially in the acidic soil. Phenanthrene was degraded to a greater extent in UG14Lr-inoculated or corn-planted soils than uninoculated and unplanted soils. The spiked phenanthrene was completely removed within 70 days in all the treatments in slightly alkaline soil. However, in acidic soil, complete phenanthrene removal was found only in the corn-planted treatments. The shoot and root lengths of corn grown in UG14Lr-inoculated soils were not different from those in non-inoculated soil between the treatments. The results showed that in unplanted soil, low pH adversely affected the survival and phenanthrene degradation ability of UG14Lr. Planting of corn significantly enhanced the survival of UG14Lr cells in both the bulk and rhizospheric soil, and this in turn significantly improved phenanthrene degradation in acidic soil. Re-inoculation of UG14Lr in the acidic soil increased the number of UG14Lr cells and enhanced phenanthrene degradation in unplanted soil. However, in corn-planted acidic soils, re-inoculation of UG14Lr did not further enhance the already active phenanthrene degradation occurring in both the bulk or rhizospheric soils.

KEY WORDS: acidic soil, corn, phenanthrene, phytoremediation, plant-microbe interaction, *Pseudomonas* sp. UG14Lr

INTRODUCTION

Phytoremediation is a cost-effective method for the removal of polycyclic aromatic hydrocarbons (PAHs) from soil. Plants may support bacterial growth by providing a hospitable microenvironment in the rhizosphere and this in turn increases the number of PAH degraders in soil (Mueller and Shann 2006). Enhanced PAH degradation has been reported

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under both single plant (Huang *et al.* 2004; Hynes *et al.* 2004; Mueller and Shann 2006) and mixed plant (Joner *et al.* 2001; Johnson *et al.* 2005; Maila *et al.* 2005; Xu *et al.* 2006) cultivation conditions. Bacterial inoculation into planted soil has been reported to further enhance PAH removal (Ankhina *et al.* 2004). It has been suggested that the main challenge for PAH degraders inoculated into soil is the extent of their survival and competitiveness relative to indigenous microorganisms (Errampalli *et al.* 1998).

The soil in areas along the coastal plains of several countries in southeast Asia is mainly acidic with high sulfur content (Bronswijk *et al.* 1995; Husson *et al.* 2000; Shamshuddin *et al.* 2004). The acidity may pose additional challenges in efforts to remediate PAH contaminants from soil. For example, in one study, the extent of anthracene being degraded by *Sphingomonas paucimobilis* strain BA2 in acidic soil (pH 5.2) was only 35%, while anthracene degradation was 88% within 10 days in neutral soil (pH 7.0) (Kästner *et al.* 1998). In another study, low soil pH (3.2–4.8) was reported to retard plant growth by causing deficiency of N, P and Mo, increasing the amount of H^+ and phenolic acid in soil, and mobilizing toxic metals such as Al from soil (Kidd and Proctor 2001). Reduced plant growth can adversely affect the capacity and effectiveness of phytoremediation in conjunction with bacterial inoculants.

Degradation of PAHs by bacterial inocula has been reported in pot studies using soil with relatively neutral pH values ranging from 6.2 to 7.6 (Anokhina *et al.* 2004; Huang *et al.* 2004; Johnson *et al.* 2004). A few studies have examined PAH biodegradation in acidic soil with pH values ranging from 2 to 5 (Stapleton *et al.* 1998; Uyttebroek *et al.* 2007), but these studies used indigenous bacteria or mixed enrichment cultures from acidic soil. Soil pH is known to affect PAH biodegradation (Kästner *et al.* 1998; Somtrakoon *et al.* 2008). However, it is not known if the presence of plants can mitigate the effect of low pH on PAH degradation by a competent microbial inoculum. Also, in any bioaugmentation studies, the competitiveness of the microbial inoculum relative to indigenous microorganisms needs to be examined.

The objective of this study was to assess if a growing plant can enhance the survival of, and phenanthrene degradation by, inoculated bacteria in two soils, one of slightly alkaline pH and the other acidic pH. In an earlier study, phenanthrene biodegradation increased significantly (89–90% of initial concentration of 100 mg/kg) in acidic soil either planted with corn alone or planted with corn and inoculated with *Pseudomonas putida* MUB1. However, the survival of MUB1 was not followed during the course of the experiment (Chouychai *et al.* 2009). The plant used in this study was corn (*Zea mays*), which has been shown to be tolerant of phenanthrene and other petroleum compounds in acidic soil (Chouychai *et al.* 2007). The bacterium used was *Pseudomonas* sp. UG14Lr which has been marked with *luxAB* genes from *V. harveyi* (Weir *et al.* 1995). The availability of the bioluminescent *luxAB* marker in UG14Lr allowed us to follow its fate unambiguously after inoculation. The parent strain (*Pseudomonas* sp. UG14) was initially isolated for its ability to mineralize phenanthrene (Providenti *et al.* 1995). Strain UG14Lr has been shown to mineralize phenanthrene in creosote-contaminated soil (Weir *et al.* 1996) and soil slurries (Weir *et al.* 1995).

MATERIALS AND METHODS

Soil Preparation

Two different soils were used in this study. The first was a slightly alkaline agricultural soil with no previous history of hydrocarbon contamination. It was collected from the

Agroforestry Research Station at University of Guelph, Guelph, Ontario, Canada. The soil was air-dried at room temperature (25°C) for at least 24 h to constant weight before use. A sample of this soil was sent to the Analytical Services Laboratory, University of Guelph for physical and chemical analyses.

The second was an acidic agricultural soil with no previous history of hydrocarbon contamination. It was collected from a forested area in Trat Province, Thailand. The soil was kept at room temperature (28–31°C) in plastic bags and was air-dried at room temperature (28–31°C) for at least 24 h to constant weight before use. A sample of the soil was sent to the Department of Land Development, Ministry of Agriculture and Cooperatives, Chonburi Branch, Thailand for chemical and physical characterization.

Both soils were used for germination of corn seeds and in pot experiments. For experiments, each soil was mixed with phenanthrene (either from Sigma or Fluka) dissolved in acetone to a final concentration of 100 mg/kg dried soil.

Experimental Design

The following five experimental treatments were used initially for each of the two soils: (a) soil inoculated with *Pseudomonas* sp. UG14Lr cells, (b) soil grown with corn only, (c) soil grown with corn and inoculated with UG14Lr cells, (d) soil which did not receive either corn or bacterial inoculum (untreated control), and (e) soil which received the modified mineral salt medium (MSM) only (MSM control). Each treatment was done in triplicate. In treatments (b) and (c) which were planted with corn, samples were taken from both the bulk and rhizospheric soil for analysis.

During the course of experiment with the acidic soil, it became apparent from analysis that UG14Lr did not survive well. Therefore, we added two experimental treatments in which fresh cultures of *Pseudomonas* sp. UG14Lr were re-inoculated into planted (f) and unplanted (g) acidic soil on day 20, in addition to the initial inoculation on day 0.

BACTERIAL PREPARATION

This was done according to Weir *et al.* (1996) with minor modifications. A loopful of *Pseudomonas* sp. UG14Lr cells growing on a TSA plate supplemented with 50 μg/ml each of ampicillin and rifampicin was transferred to 50 ml of a GY medium consisting of mineral salt medium (Weir *et al.* 1996) plus 1.0% (w/v) glycerol and 0.2% (w/v) yeast extract. The culture was shaken at 200 rpm, 30°C for 24 h. Five ml of this culture were inoculated into 50 ml of GY medium supplemented with 100 mg/l phenanthrene, and the culture was shaken at 200 rpm at 30°C for 24 h. UG14Lr cells were cultured in GY medium + 100 mg/l phenanthrene for 24 h before being inoculated to the soil. Cell were harvested by centrifugation (4,000 g for 10 min at 4°C) and then resuspended in sterile MSM medium for 10^{10} cell/ml

Plant Preparation

Hybrid sweet corn vr. 132A white seeds from Stokes Seed Ltd. (Thorold, Ontario, Canada) were used for the slightly alkaline soil in Canada, while sweet corn white seeds from Yongseng Chemical Agriculture Ltd. (Nonthaburi Province, Thailand) were used for

the acidic soil in Thailand. The seeds were immersed in tap water for 3 h, and then sown in 100 mg/kg phenanthrene-contaminated soils.

Pot Experiment

Twelve day-old corn seedlings were planted in soil containing 100 mg phenanthrene per kg dry soil at one plant per pot. The diameter of the pot was 7 in, with each pot holding 1 kg dry weight of soil. *Pseudomonas* sp. UG14Lr cells were inoculated into soil to final concentrations of about 10^7 CFU/g dry soil and mix thoroughly with a digger. The pots were kept at 24°C during the day and 21°C at night, exposed to natural sunlight, and watered every day. The experiment was continued until all the added phenanthrene had disappeared from the soil. This took about 70 days. The experiment was conducted in triplicate.

At each 10-day sampling period, the corn plants were removed and the pots terminated. Plant growth was followed by measuring the shoot and root lengths of corn plants with a ruler.

Soil Analysis

One g of soil sample was collected from each treatment every 10 days for various analyses. For non-planted pots, the samples were taken at about 7 cm depth. For planted pots, the corn plant was removed first and the rhizospheric soil collected. The soil remaining in pot was transferred to another empty pot, mixed and then sampled.

The total numbers of *Pseudomonas* sp. UG14Lr cells and aerobic heterotrophic bacteria were determined according to Weir *et al.* (1996) and Errampalli *et al.* (1998) with some modifications. Briefly, the 1-g soil sample was placed in 9.5 ml of 0.1% (w/v) sodium pyrophosphate solution and shaken at 180 rpm for 2 h. The suspension was serially diluted in sodium pyrophosphate solution and plated on TSA media for total aerobic heterotrophic bacterial count and TSA media + 50 μg/ml ampicillin + 50 μg/ml rifampicin + 40 μg/ml methyl 1-(butylcarbomoyl)-2-benz-imidazolecarbamate (Aldrich, 95%) for *Pseudomonas* sp. UG14Lr count. Plates were incubated for 2 days and colonies appearing on agar were counted. UG14Lr colonies were also confirmed by their bioluminescent character due to the presence of the *luxAB* genes (Flemming *et al.* 1994). This was done by adding drops of the 0.1% *n*-decanal substrate (Fisher Scientific, 95%, dissolved in 100% ethyl alcohol) to the lid of the Petri plate and then observing and quantifying light emitted using a Night Owl, EG&G Berthold Molecular Light Imager (Fisher Scientific).

The amount of phenanthrene remaining in soil was determined by GC according to Chouychai *et al.* (2009). Briefly, one g of soil was collected, mixed with an equal amount of anhydrous Na_2SO_4, and subjected to Soxhlet extraction. The phenanthrene content in soil extracts was analyzed by GC-FID. Compounds were separated on a 30 m column (DB-TPH) with 0.32 mm internal diameter and a film thickness of 1.0 micron (J & W Scientific, part number 123–5033) as described in Chouychai *et al.* (2009).

Statistical Analysis

Two-way ANOVA was used to test for significant differences between treatments followed by Tukey's test.

RESULTS

Soil Properties and Plant Health

The slightly alkaline (pH 8.1) Canadian agricultural soil was a very fine sandy loam which contained 0.13% nitrogen, 10 ppm phosphorous, 54 ppm potassium, and 2.8% organic matter. The acidic Thai soil had a pH of 3.3, a low organic content (0.99% w/w) and an electrical conductivity of 0.01 dS/m. The soil contained 0.049% nitrogen, 12 ppm phosphorus, and 22 ppm potassium (Chouychai *et al.* 2007).

The shoot length of all the corn plants increased steadily before leveling off at 40–60 days. In phenanthrene-free slightly alkaline control soil, the corn shoots grew at a slightly faster initial rate and achieved greater length than those of corn grown in phenanthrene-spiked soils (Figure 1a). In the acidic phenanthrene-free control soil, the corn shoots grew faster in the first 20 days, but then leveled off and achieved shorter lengths than those of corn in the UG14Lr-inoculated phenanthrene-spiked soils (Figure 1c). Corn plants grown in phenanthrene-spiked soil, with or without the UG14Lr inoculum, showed the presence of yellow leaves by day 20. This was considerably earlier than the non-spiked control treatment in which the corn plants showed the presence of yellow leaves on day 40.

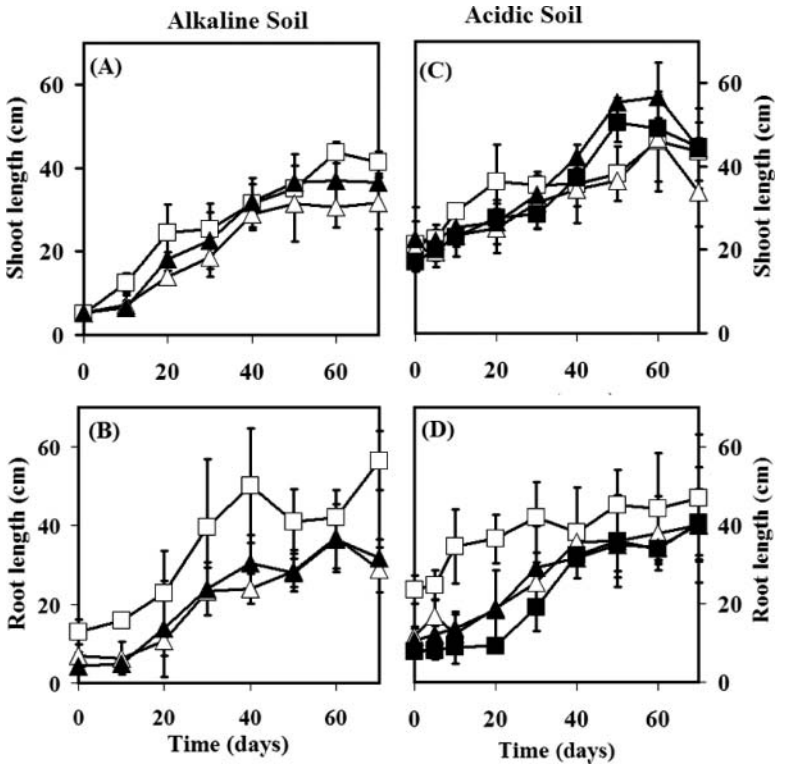

Figure 1 Shoot length of corn plants grown in slightly alkaline (a) and acidic soil (c), and root length of corn plants grown in slightly alkaline soil (b) and acidic soil (d) with or without inoculation of *Pseudomonas* sp. UG14Lr spiked with 100 mg/kg phenanthrene compared with corn grown in non-spiked soil. Symbols: □ non-spiked soil, △ phenanthrene-spiked soil, ▲ UG14Lr inoculated and phenanthrene-spiked soil, ■ UG14Lr re-inoculated and phenanthrene-spiked soil.

The roots of corn plants in the two phenanthrene-free control soils grew longer throughout the experimental period than those of corn grown in phenanthrene-spiked soils (Figure 1b and 1d). On day 70, the roots of corn grown in the phenanthrene-free slightly alkaline control soil were about 40%, and statistically significantly ($P < 0.05$), longer than those of corn grown in the presence of phenanthrene (Figure 1b). Addition of the UG14Lr inoculum did not appreciably affect the root length in phenanthrene-spiked soils. None of the plants had flowered at the end of experiment on day 70. It should be noted that the roots of corn plants grown in slightly alkaline soil looked more healthy and fibrous than that of corn grown in acidic soil.

Phenanthrene Biodegradation in Soil Microcosms

In slightly alkaline soil, phenanthrene was degraded rapidly by more than 50% within the first 10–20 days in all treatments, except the untreated control, after which the rate of degradation leveled off (Figure 2a and 2b). By day 70, more than 98% of phenanthrene was degraded in all except the control treatment (Figure 2a). In the untreated control soil, phenanthrene was not degraded in the first 10 days. Thereafter, phenanthrene was degraded steadily, such that by day 40, about 25% remained in the soil. The residual phenanthrene was not degraded to the same extent as in the other treatments, and on day 70, about 8% remained in the soil (Figure 2a). Addition of the UG14Lr inoculum enhanced phenanthrene degradation such that more than 40 and 80% was degraded by 10 and 20 days, respectively.

In acidic soil, more than 50% of the phenanthrene was degraded within the first 10 days (Figure 2d). By day 70, more than 98% of phenanthrene was degraded in all except the control treatments (Figure 2d) and in the UG14Lr-inoculated but unplanted treatment (Figure 2e). In the unplanted acidic soil, phenanthrene was degraded slowly in the first 30 days. More than 50% of phenanthrene was degraded after 50, 40, and 30 days in the untreated control, MSM control, and UG14Lr inoculated soil, respectively. By day 70, about 40% phenanthrene remained in the untreated control and MSM control (Figure 2d), while about 20% phenanthrene remained in UG14Lr inoculated soil (Figure 2e).

In corn-planted slightly alkaline microcosms, phenanthrene was degraded rapidly in both the bulk and rhizospheric soils compared to the untreated or media controls (Figure 2a). Addition of the UG14Lr inoculum to the corn-planted soil microcosm further enhanced the initial rate of phenanthrene degradation in both the bulk and rhizospheric soils (Figure 2b), relative to those in the corn-planted microcosm which did not receive the UG14Lr inoculum (Figure 2a). Notably, more than 50% of the phenanthrene was degraded by day 10 in the slightly alkaline soil microcosm which received the mineral salt medium only (Figure 2a). However, in subsequent sampling days, the amount of phenanthrene remaining was much higher than the other experimental treatments, except for the untreated control. Thus, we surmised that the low phenanthrene measurement for the day 10 sample may be an experimental error.

In corn-planted acidic soil, phenanthrene was degraded rapidly in both the bulk and rhizospheric soil by more than 70 and 90% in the first 10 and 20 days, respectively (Figures 2d and 3a). Because of this fast initial rate, addition of UG14Lr did not seem to further enhance phenanthrene degradation (Figure 2e). Not surprisingly, re-inoculation of UG14Lr on day 20 also did not significantly alter the rapid phenanthrene biodegradation profile (Figure 3a). In unplanted acidic soil inoculated with UG14Lr, phenanthrene degradation was slower than corn-planted acid soils (Figure 3a). UG14Lr re-inoculation on day 20 did not significantly increase phenanthrene degradation (Figure 3a). In any event, by day

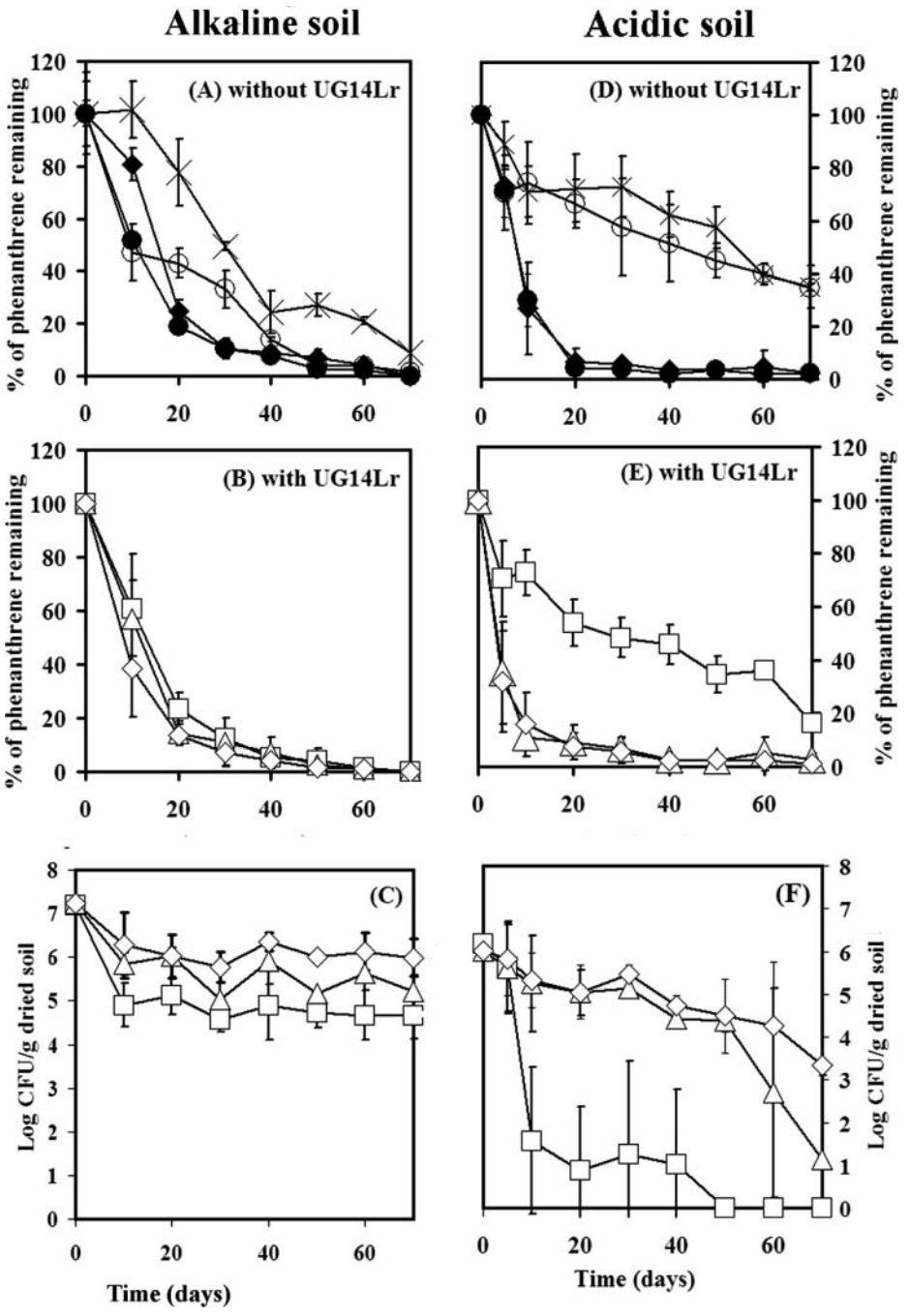

Figure 2 The percentage of phenanthrene remaining in slightly alkaline soil planted or unplanted with corn and uninoculated (a), inoculated with *Pseudomonas* sp. UG14Lr (b), in acidic soil planted or unplanted with corn and uninoculated (d), inoculated with *Pseudomonas* sp. UG14Lr (e), the total number of *Pseudomonas* sp. UG14Lr cells in slightly alkaline soil planted or unplanted with corn (c), or in acidic soil planted or unplanted with corn (f). The starting concentration of phenanthrene was 100 mg/kg dried soil. Symbols: x untreated control, ○ MSM control, ♦ corn planted bulk soil, • corn rhizosphere, □ UG14Lr inoculated soil, △ UG14Lr inoculated + corn planted bulk soil, ◇ UG14Lr inoculated + corn planted rhizosphere

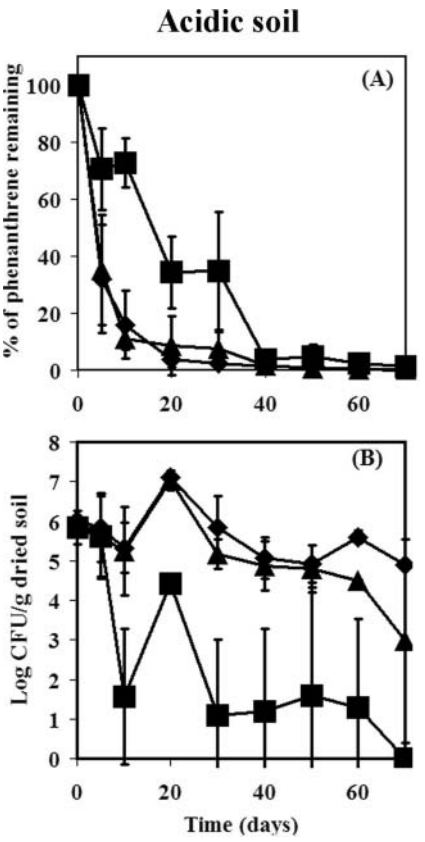

Figure 3 The percentage of phenanthrene remaining (a) and the total number of *Pseudomonas* sp. UG14Lr cells (b) in acidic soil planted or unplanted with corn and re-inoculated with *Pseudomonas* sp. UG14Lr. The starting concentration was 100 mg/kg dried soil. Symbols: ■ UG14Lr re-inoculated soil, ▲UG14Lr re- inoculated + corn planted bulk soil, ♦ UG14Lr re-inoculated + corn planted rhizosphere.

40 almost all the phenanthrene was degraded. This is considerably faster than that in the unplanted microcosm which received only one UG14Lr inoculum on day 0 (Figure 2e).

Bacterial Numbers in Soil Microcosms during Phenanthrene Degradation

In unplanted microcosms, UG14Lr cells survived considerably better in the slightly alkaline soil than in acidic soil. For example, in the slightly alkaline soil, the number of UG14Lr cells decreased from log 7.2 to log 4.9 CFU/g dried soil within 10 days after inoculation (Figure 2c). Thereafter, the numbers fluctuated slightly and stayed slightly under log 5 CFU/g dried soil at 70 days. In contrast, in acidic soil, UG14Lr numbers dropped rapidly from log 6 to log 1.57 from day 0 to day 10, and then fluctuated between log 1–2 CFU/g dried soil up to 40 days before becoming undetectable by 50 days (Figure 2f).

The presence of corn plants enhanced the survival of UG14Lr cells in both the bulk and rhizosphere soil compared to unplanted soil (Figure 2c and 2f). This effect was

consistent throughout the 70-day experimental period and was particularly pronounced in acidic soil. UG14Lr numbers in corn planted acidic soil decreased slowly from log 6.0 on day 0 to log 4.37 and log 4.49 CFU/g dried soil on day 50 in both the bulk and rhizospheric soils. UG14Lr numbers in the bulk soil then decreased rapidly to log 1–3 CFU/g dried soil between days 60–70, while the number in the rhizospheric soil ranged between log 3–4 CFU/g dried soil until the end of the experiment (Figure 2f). Thus, the general trend was that the rhizosphere harbored more UG14Lr cells than the bulk soil in the corn-planted microcosm.

Re-inoculation of UG14Lr on day 20 transiently increased the number of UG14Lr cells in both planted and unplanted acidic soils (Figure 3b). In unplanted acidic soil, UG14Lr number decreased rapidly from log 4.43 (after re-inoculation) on day 20 to log 1.1 CFU/g dried soil on day 30. The number subsequently ranged between log 1–2 CFU/g dried soil until becoming undetectable on day 70. In corn-planted acidic soil, the UG14Lr numbers decreased from log 7.03 and log 7.09 on day 20 (after re-inoculation) to log 5.17 and 5.83 CFU/g dried soil on day 30 for the bulk and rhizospheric soil, respectively. The number of UG14Lr cells ranged between log 4.5–5 CFU/g dried soil until day 60. On day 70, the number of UG14Lr cells in the corn-planted bulk soil decreased to log 2.97 CFU/g dried soil while that in the corn-planted rhizospheric soil (log 4.9 CFU/g dried soil) remained similar to that on day 60 (Figure 3b).

DISCUSSION

The two key biological requirements for effective phytoremediation of PAH-contaminated soil are the presence of robust PAH-tolerant plant and competent PAH-degrading microorganisms. In previous studies, corn was shown to be a PAH-tolerant plant (Chouychai *et al.* 2007) with the ability to enhance phenanthrene biodegradation in contaminated acidic soil (Chouychai *et al.* 2009). Planting corn alone or co-inoculation with *Pseudomonas putida* MUB1 could remove 90% of the phenanthrene (initial concentration = 100 mg/kg) within 60 days. However, in that study, the survival of MUB1 was not monitored and thus the relationship between MUB1 survival and phenanthrene degradation was not known (Chouychai *et al.* 2009).

In the present study, the effectiveness of corn in enhancing phenanthrene biodegradation was compared between a slightly alkaline soil and acidic soil. In addition, we used the phenanthrene-mineralizing bacterium *Pseudomonas* sp. UG14Lr which has been marked with *luxAB* genes (Weir *et al.* 1995) to facilitate its tracking after inoculation. Moreover, we were particularly interested to assess if UG14Lr could survive and degrade phenanthrene effectively in both slightly alkaline and acidic soils in the absence or presence of corn plant.

Our results showed that the presence of corn plant increased phenanthrene biodegradation in both the slightly alkaline and acidic soils. Moreover, the presence of the corn plant improved UG14Lr survival in both soil types. This beneficial effect was more pronounced in the rhizosphere than the bulk soil and was greater in acidic soil than slightly alkaline soil. For example, in unplanted slightly alkaline soil microcosm, the number of UG14Lr cells decreased from log 7.2 to log 4.9 CFU/g dried soil in the first 10 days. In comparison, in corn-planted soil, the decreases in the number of UG14Lr cells, down to log 5.84 and log 6.28 CFU/g dried soil in the bulk and rhizospheric soil, respectively, were not as drastic as that seen in the unplanted soil (Figure 2c).

As shown in control pots, both soils contained indigenous microorganisms able to degrade phenanthrene. Addition of a competent UG14Lr inoculum further increased

phenanthrene biodegradation in non-planted slightly alkaline soil but not in non-planted acidic soil. This corresponds to the relatively good survival of UG14Lr cells in the slightly alkaline soil, but not acidic soil. The results showed that UG14Lr did not work well in non-planted acidic soil. This is not surprising considering that *Pseudomonas* sp. UG14Lr is a derivative of *Pseudomonas* sp. UG14 which was isolated from soil of relatively neutral pH (7.4) (Providenti *et al.* 1995).

Interestingly, the presence of corn plant significantly enhanced UG14Lr survival in acidic soil and this in turn enhanced phenanthrene biodegradation rate in this soil. The beneficial effect of corn plant was much more apparent in acidic soil than slightly alkaline soil, mainly because UG14Lr survived so poorly in non-planted acidic soil. In contrast, UG14Lr survived well in the slightly alkaline soil, and thus the presence of corn plant, while helpful, did not lead to further significant enhancement in UG14Lr survival.

Bioaugmentation involves the inoculation of a suitably competent microbial inoculant to a site to increase the effectiveness of bioremediation. Our results underscore the importance of selecting competent microorganisms already adapted to the conditions prevailing at the site of intended release to allow optimal bioremediation to proceed. In the absence of a suitably adapted microbial inoculum, the presence of an adapted plant can enhance the survival of the less adapted microbial inoculant and partially improve phenanthrene degradation.

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