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INTRODUCTION

Land farming, for the purpose of bioremediation, refers traditionally to the spreading of contaminated soil, sediments, or other material over land; mechanically mixing it; incorporating various amendments, such as fertilizer or mulch; and sometimes inoculating with degradative microorganisms. In general, living plants are not involved in the process. Populations of bacteria added to soils often decline rapidly and become metabolically inactive. To efficiently degrade contaminants, microorganisms must be metabolically active. Thus, a significant obstacle to the successful use of microorganisms for environmental applications is their long-term survival and the expression of their degradative genes in situ.

In bulk soil, carbon is often a limiting resource and its absence can lead to a failure to bioremediate (Boething & Alexander 1979, Bolton et al. 1992, Goldstein et al. 1985, Schmidt & Alexander 1985). Rhizosphere microorganisms are known to be more metabolically active than those in bulk soil, because they obtain carbon and energy from root exudates and decaying root matter (Bolton et al. 1992). In addition to being more active, rhizosphere populations are more abundant, often containing 10^8 or more culturable bacteria per gram of soil, and bacterial populations on the rhizoplane can exceed 10^9/g root (Fredrickson & Elliott 1985, Bolton et al. 1992).

Many of the critical parameters that influence the competitive ability of rhizosphere bacteria have not been identified, but microorganisms have frequently been introduced into soil (bioaugmentation) as part of routine or novel agronomic practices. These include the application of *Rhizobium* sp. to improve nitrogen fixation in leguminous plants, biocontrol of root pathogens via inoculation with a variety of bacteria, and inoculation to stimulate root and plant growth (Bolton et al. 1992, De Freitas & Germida 1991, Macdonald 1989, Sivan & Chet 1992, Zablotowicz et al.

1991, 1992). However, the use of rhizosphere bacteria and their in situ stimulation by plant roots for degrading organic contaminants has received little attention. The few studies that have been published demonstrate that the use of plants enhances the rate of loss of organic contaminants (Aprill & Sims 1990, Hsu & Bartha 1979, Knaebel 1992, Reddy & Sethunathan 1983, Walton & Anderson 1990).

Published studies have demonstrated the feasibility of using rhizobacteria (*Pseudomonas putida*) for the rapid removal of chlorinated pesticides from contaminated soil (Short et al. 1990), and to promote germination of radish seeds in the presence of otherwise phytotoxic levels of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) (Short et al., 1990), and phenoxyacetic acid (PAA) (Short et al., 1992). The present investigation was undertaken to determine if these strains (*Pseudomonas putida* PPO301/pRO101 and PPO301/pRO103) could be used to bioremediate 2,4-D-amended soil via plant-microbe bioaugmentation.

Methods

Microcosms were prepared in 50 ml sterile, disposable, polypropylene centrifuge tubes containing 50 g (wet weight) of Burbank sandy loam (see Cataldo et al. 1990 for soil characteristics) at 30% moisture. The soil treatments were prepared in bulk, in one quart canning jars and uniformly mixed on a roller mill, prior to dispensing into tubes. For the initial 30 day greenhouse trial, the following treatments were employed: 0 (control) or 500 µg/g 2,4-D; uninoculated or uniformly inoculated with an equal mixture of *Pseudomonas putida* strains PPO301/pRO101 and PPO301/pRO103{both are Nal^r Tc^r} (Harker et al. 1989, Short et al. 1990) to 10^9 colony-forming units (cfu)/g; unplanted or planted with three seeds each of *Triticum aestivum* L. cv. Edwall) inserted three cm into the soil.

A second microcosm-based experiment utilized a soil-overlay inoculation method to examine the colonization ability of the *Pseudomonas putida* strains. Burbank sandy loam soil (non-sterile) was amended to either 0 or 500 µg/g 2,4-D and brought to 30% moisture with sterile distilled water, and then 40 g was added to microcosm tubes. This layer of soil was overlain with

10 g of moist sterile soil (= uninoculated) or 10 g of inoculum. The inoculum consisted of sterilized Burbank sandy loam (autoclaved 1 h on each of 3 separate days), brought to 30% moisture and inoculated with an equal mixture of *P. putida* PPO301/pRO101 and PPO301/pRO103, incubated 48 hours at 30°C (population of 10^9 cfu/g determined by viable counts) prior to dispensing into microcosm tubes. The microcosms either remained unplanted or received three wheat seeds inserted into the upper (overlay) soil layer.

Microcosms were analyzed for plant top and root dry weight, soil and rhizosphere counts of inoculum strains and populations of total soil heterotrophic bacteria. Visible estimates of root length were made. Analysis of 2,4-D in soil was performed via high-performance liquid chromatography (HPLC) according to Short et al. (1990).

RESULTS

In uninoculated, 2,4-D-amended microcosms wheat seeds failed to germinate. Microbial inoculation protected the wheat seeds in the 500 µg/g 2,4-D-amended treatments. However, the seeds germinated later and grew more slowly and produced less shoot, but more root dry matter than plants from the unamended control microcosms as measured after 30 days (see Figure 1). The roots near the crowns of these plants were visibly deformed and distorted; the roots were also shorter than the roots of control plants. The roots of the control plants extended to the bottom of the microcosm tubes (ca. 60 mm) within the first 2 weeks and continued elongating, forming compacted root masses at the bottoms of the microcosm tubes. The longest roots in the experimental treatment (inoculated, 500 µg/g 2,4-D) never reached the bottom of the microcosms (i.e. < 60 mm) In inoculated treatments, 2,4-D was reduced 99% in 30 days, i.e., from 500 µg/g to 5 µg/g. There was not a measurable difference in the level of 2,4-D remaining in the inoculated microcosms that contained wheat plants or those without plants (i.e. ca. 5 µg/g 2,4-D remained). In the uninoculated treatments, the indigenous soil microflora decreased the initial level of 2,4-D from 500 µg/g to only 250 µg/g (50%) after 30 days.

In the overlay inoculation trial wheat seeds germinated readily in all treatments; however, in uninoculated, 2,4-D-amended treatments the seedlings were killed within ca. 2 weeks of germination as a result of 2,4-D toxicity. Viable counts of root and soil samples from inoculated control treatments (i.e., 0 μg/g 2,4-D) and inoculated experimental (500 μg/g 2,4-D) (see Table 1) indicated that the inoculum readily colonized the wheat roots. Unlike the previous experiment there was not a significant difference in shoot dry weights between control microcosms (0 μg/g 2,4-D, either inoculated or uninoculated) and experimental microcosms (500 μg/g 2,4-D, inoculated). However, there was a significant difference in root growth between the two treatments. In control microcosms, the roots reached the bottom of the tubes (as observed in the previous experiment); in experimental treatments (2,4-D, inoculated), despite being well colonized the roots were confined to the upper inoculum layer and the first ca. cm of the 2,4-D-containing layer. The root systems of these plants were grossly deformed. The level of 2,4-D remaining in these microcosms after 30 days was ca. 300 μg/g (whether inoculated or uninoculated).

DISCUSSION

The use of rhizosphere-competitive bacteria for bioremediation offers several advantages. Many organic contaminants cannot serve as sole carbon and energy sources (or are not present in high enough concentrations to do so) and are degraded only when additional electron donors are supplied. However, rhizosphere bacteria derive most substrates and carbon from the plant root and remain metabolically active for extended periods, and thus they can continue to degrade the contaminants for an extended period of time. As plant roots grow through the soil, microorganisms continuously colonize the growing root tips. Depending on the plant species, root growth can be extensive both horizontally and vertically. It is not uncommon for roots of legumes and plants from arid regions to penetrate to 10-15 m below the soil surface. Grass species typically have dense rooting patterns and can spread by rhizomes. For example, it has been estimated that a single wheat plant (*Triticum aestivum*) can produce a total root length approaching

71,000 m, which constitutes a large surface area dispersed throughout the soil (Pavlychenko 1937). The few studies that have been published demonstrate that the use of plants enhances the rate of loss of organic contaminants (Aprill & Sims 1990, Hsu & Bartha 1979, Knaebel 1992, Reddy & Sethunathan 1983, Walton & Anderson 1990). These studies relied upon the rhizosphere stimulation of the indigenous microflora and dealt with relatively low concentrations of compounds.

Uniform soil inoculation to 10^9 cfu/g, as in the first microcosm trial described here or in previous studies (Short et al. 1990, 1992), is readily achievable in the laboratory, but it is unrealistic for the field. For field-scale applications of rhizosphere bioaugmentation some form of seed/plant inoculation, such as by seed coating or in furrow inoculation will be required. Therefore aggressive, rhizosphere-competent, root-colonizing organisms will be necessary.

In both trials inoculation was required for wheat seed germination and growth in the presence of 500 µg/g 2,4-D. In the absence of 2,4-D the *P. putida* inoculum colonized roots in the control microcosms at ca. 10^6 cfu/g dry root in the presence of ca. 10^8 cfu/g (soil) of competing heterotrophic bacteria (Table 1). However, in the experimental treatments an inoculum population of 10^7 cfu/g root was apparently insufficient to allow wheat roots to penetrate more than a few mm into the 2,4-D-containing soil layer. The distorted and deformed root systems were confined to the inoculum layer and the upper few mm of the 2,4-D layer. The overall level of 2,4-D to which the plant roots were exposed as they attempted to penetrate downward was high (ca. 300 µg/g), resulting in the distortion of the root system and lack of vertical root growth. The differences observed between root growth in the two microcosm trials can be attributed to the mode of inoculation. In the uniformly inoculated trial the 2,4-D was degraded globally throughout the microcosm; the lowered levels of 2,4-D allowed for more extensive vertical root growth. By contrast, in the overlay trial the inoculum/roots were exposed to a steep vertical gradient of 2,4-D.

CONCLUSIONS

The current experiments demonstrate that bioaugmentation with rhizosphere-competent bacteria, capable of degrading the herbicide 2,4-D can protect seeds, allowing germination and plant growth to proceed in the presence of an otherwise phytotoxic level of herbicide. Because 2,4-D is a synthetic auxin, even modest amounts can affect root growth and morphology. Plant-microbe bioaugmentation should work more effectively with other organic pollutants, especially those that are not phytotoxic and do not have plant growth regulatory effects.

ACKNOWLEDGMENT

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LIST OF TABLE AND FIGURE CAPTIONS

Figure 1. Mean wheat plant shoot and root dry weights following a 30 day greenhouse trial; seeds failed to germinate in uninoculated microcosms.

TABLE 1. Viable counts of inoculum strains and heterotrophic bacteria from upper and lower roots and soil of overlay inoculated microcosms.

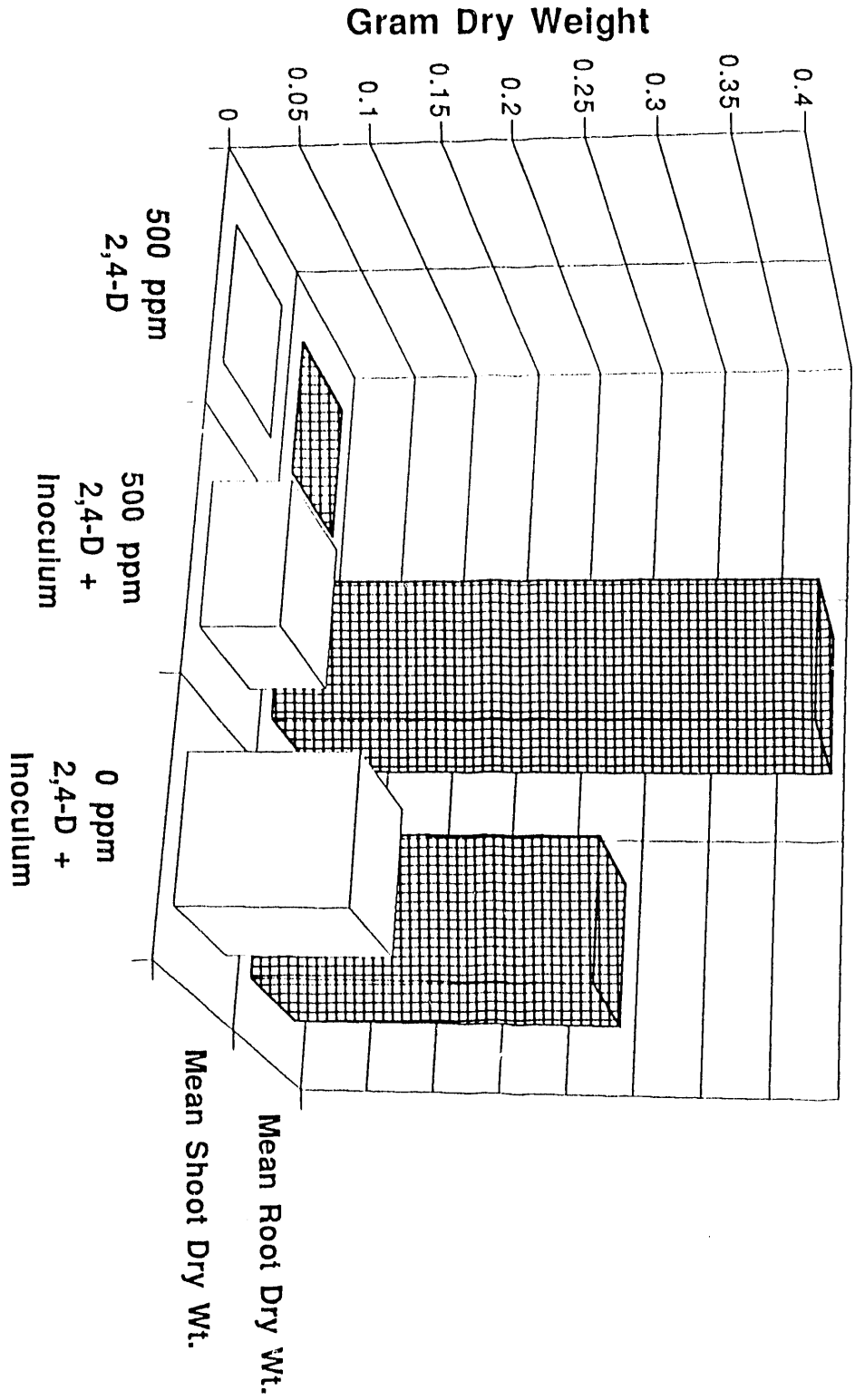
Gram Dry Weight
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
500 ppm 2,4-D
500 ppm 2,4-D + Inoculum
0 ppm 2,4-D + Inoculum
Mean Root Dry Wt.
Mean Shoot Dry Wt.

TABLE 1.

	Inoculum[a]		Total Heterotrophs[b]	
Inoculated Control Plants	Root counts[c] cfu/g	Soil Counts[d] cfu/g	Root counts cfu/g	Soil Counts cfu/g
upper	1×10^8	8×10^6	NT[e]	8×10^7
lower	4×10^6	1×10^3	NT	3×10^7
Inoculated/500 µg/g 2,4-D				
upper	7×10^7	1×10^7	NT	3×10^8
lower	NA[f]	$< 10^1$	NA	6×10^7

(a) Inoculum plated on media containing NaI (100 µg/ml) and Tc (20 µg/ml)
(b) Plated on TSA
(c,d) Root and soil counts on dry weight basis
(e) NT = not tested
(f) NA = not applicable

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