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FLUORESCENT, ROOT-COLONIZING RECOMBINANT BACTERIA TO ENHANCE THE RHIZOSPHERE DEGRADATION OF TCE

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ABSTRACT: Remediation of trichloroethylene (TCE) and other chlorinated solvents via poplar trees has been shown at various field sites. The ultimate fate of the contaminants is still unclear and volatilization to the atmosphere appears to be dominant. The degradation of TCE and via in situ rhizoremediation using recombinant bacteria is an enhanced option for phytoremediation. This plant-bacteria degradation is favorable as it limits the potential entry of the contaminant into the food chain or transfer to other media (air). Microorganisms isolated from poplar trees roots were genetically modified to carry toluene *ortho*-monoxygenase (TOM) enzymes to degrade chlorinated solvents. Increased TCE degradation was observed in the rhizosphere of inoculated plants. However, the plant-microbe symbiosis is not well understood. To further investigate this interaction, these same host organisms have been tagged with the gene, *gfp* resulting in constitutive expression of the gene in the bacteria chromosomal DNA. Green fluorescent protein is a useful method for tracking the introduced bacteria. The protein can be used to detect the activity and growth patterns of recombinant microorganisms. The stable recombinant microorganisms were reintroduced to the rhizosphere. Using MPN plating and epifluorescent microscopy of the colonized roots, the recombinants were shown to survive for >28 days under laboratory conditions. Following a four-week growth period, roots had 2 x 10^4 to 26 x 10^4 colonies per inch. Growth patterns along the poplar roots under different conditions were also analyzed and individual bacteria and colonies can be visualized. The recombinant bacteria have been used to study transport of the bacteria to other plant species. Through using the *gfp*- and TOM- recombinants the degradation of contaminants (TCE) and the interactions between plant and bacteria facilitating degradation are now better understood.

INTRODUCTION

Rhizosphere degradation of contaminants is an important aspect of phytoremediation. Contaminants such as trichloroethylene (TCE) and other chlorinated solvents in soil and ground water can be influenced by phytoremediation and rhizosphere degradation. The ultimate fate of the contaminants in many phytoremediation applications is unclear and volatilization to the atmosphere appears dominant. Increased rhizosphere degradation may impact the fate of the contaminants by causing complete mineralization. This would decrease uptake of the compounds in the plant, thus decreasing plant tissue concentrations and volatilization to the atmosphere. The rhizosphere is the root zone area directly influenced by the plant and roots. In the rhizosphere, there are increased density and activity among the microorganism cultures that interact with the roots. The plant roots exude short chain organics and phenolics, which are a source of root lubrication and energy for the microbial populations colonizing at the root interface (Schnoor, 1997). This symbiotic relationship is beneficial

to both organisms as the plant provides a constant environment for the microorganisms and the microbes can help the plant grow. The microorganisms can aid the plants in surviving in highly contaminated environments by degrading some of the compounds. The influence of the rhizosphere and potential degradation resulting from bacteria associated with the rhizosphere has been shown in numerous studies. The combination of plants and microorganisms has been shown to degrade chlorinated benzoic acid mixtures (Siciliano and Germida, 1998a). In another study by Siciliano and Germida (1998b), it was shown that rhizosphere degradation is promoted by the inoculation of Pseudomonads into the microbial community associated with the rhizosphere of *Bromus biebersteinii*. These are examples of the potential synergistic effect of plants and bacteria, although this relationship does not always prove to be beneficial. A comparative study demonstrating the fate of ^{14}C-trichloroethylene (TCE) in the rhizosphere showed that in some cases the vegetation slightly enhanced the TCE degradation by the bacteria and other cases where there was no significant effect due to the vegetation (Anderson and Walton, 1995). In this case the plants did not effectively increase the degradation. In other instances the mineralization of atrazine was shown to be significantly less in planted bioreactors compared to unplanted bioreactors due to plant uptake exceeding degradation rates (Burken and Schnoor, 1996).

A viable method to enhance contaminant degradation in the rhizosphere is to introduce genetically engineered microorganisms (GEMs). The plant rhizosphere provides a stable environment for the microorganisms with an energy source, surface area for growth, constant pH, nutrients and moisture. This environment can be highly selective for certain organisms that can colonize roots. Previous work has shown that the presence of GEMs significantly affects the contaminant removal rate. Crowley et al. (1996) demonstrated that for a recombinant strain of *Pseudomonas fluorescens* modified to degrade 2,5-dichlorobenzoate in the rhizosphere, degradative ability intensified with the addition of plants to the reactors. Another study involving the degradation of TCE using a wheat colonizing bacterial strain demonstrated that engineered microbes could enhance the degradation of contaminants in the rhizosphere. In this study there was a significant difference in the amount of TCE remediated from the soil among recombinant strains and non-engineered strains (Yee et al., 1998). These studies demonstrate the benefits of incorporating engineered microorganisms and plants in a contaminant degradation system. Work done by Shim et al. (2000) has shown native bacterial strains from poplar trees modified to degrade TCE to be stable and competitive against non-engineered bacteria in the rhizosphere of poplar trees. This controlled environment supports the colonization, proliferation, and potential transfer of the GEMs. The potential transfer refers to inoculating the stems of the tree and following extensive root growth the bacteria potentially colonizes the entire rhizosphere. A GEM-plant system has minimal ecological impact, high contaminant removal and the potential for mineralization of the compounds making the system a desirable remediation technique.

These GEM-plant systems have benefits and potential field applications but there are issues that arise from introducing genetically modified microorganisms into the natural environment. One specific issue is the ability to monitor GEMs following release. Prior GEM studies have met with some opposition related to the use of an antibiotic resistance marker for tracking. Therefore a simple and robust monitoring method is being developed to test GEM-plant systems. The use of the *gfp* gene is an

appealing tracking method for GEMs. This gene, originally found in the jellyfish *Aequorea victoria,* codes for the green fluorescent protein (GFP). The root colonizing microorganisms used in these studies are tagged with the *gfp* gene. The gene is incorporated into the microbe's chromosomal DNA and constitutively expressed by the microorganisms (Gilbertson, 2000). The presence of a substrate to initiate fluorescence is not needed and the protein is stable and continues to fluoresce even if the cells are not viable (Lowder et al., 2000). GFP is a visual monitoring marker and can be tested and observed with minimal disturbance to the site and the cell samples. This has been shown in studies using the *gfp* gene to monitor pseudomonads on barley seeds (Normander et al., 1999, and Tombolini et al., 1999) and tomato seedling roots (Bloemberg et al., 1997).

The ability to detect and enumerate GEMs in the rhizosphere without a selective antibiotic will greatly advance the use of GEMs in the environment. The use of GFP can be a valuable tool because it allows for visualization of the GEM colonization locations. With this tracking method, microorganisms that have been genetically modified to degrade contaminants can be monitored with little disturbance to the cells and plant-microorganism system.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Recombinant *gfp*-tagged poplar root-colonizing microorganisms were generated using root-colonizing host strains Pb1, Pb2, Pb3, and Pb5 originally isolated from poplar roots (Shim et al., 2000). A recombinant American Type Culture Collection (ATCC) *gfp*-tagged *Rhizobium* strain was used, as it is a known root colonizer (Gilbertson, 2000). The strains were grown on Luria-Bertani (LB) medium at 30°C (Shim et al., 2000). Kanamycin (50μg/mL) was added to the medium when growing *gfp*-tagged strains.

Growth Rate Measurement. The growth rates for the recombinant *gfp*-tagged strains were measured using a spectrophotometer. The cultures were inoculated into LB broth and the absorbance at 600 nm was measured every 30 minutes during the lag phase and every ten minutes during the exponential growth phase through the stationary and death phases. Using the data collected the growth rates of each strain were calculated.

Survival Experiment. An experiment was designed to determine the survival of recombinant *gfp*-tagged microorganisms. Recombinant *gfp*-tagged bacterial strains were inoculated onto rooting poplar trees for a growth period. Poplar cuttings were planted in sand for two weeks to induce rooting. The tree roots were rinsed with de-ionized water to remove the sand particles prior to inoculation and replanting. The recombinant strains used in the inoculation of the roots were Pb5*gfp*2-2 and *Rhizobium* 35645*gfp*2-1. The cultures were grown overnight in LB broth and then 14 ml of each was placed into sterile 15-ml screw cap test tubes. The cultures were centrifuged for five minutes to separate the cell mass and the media. The broth was poured off of the cells and the cells were washed twice with sterile 0.1 M sodium phosphate buffer (pH 7). The cells were then resuspended in the sterile 0.1 M sodium phosphate buffer (pH 7). The trees were dipped into the cell suspension and then allowed to dry for approximately 5 minutes prior to planting. Two trees were inoculated with Pb5*gfp*2-2 and two trees were inoculated with *Rhizobium* 35645*gfp*2-1. The trees were planted in eight-inch diameter containers that

were ten inches tall. The containers were filled with a 50/50 sterile mixture of potting soil and local topsoil. The soil was autoclaved for 1.5 hours. Following planting, the trees were watered daily until the top layer of soil was wet. On a weekly basis the trees were given a 10 ml supplement of ¼ strength modified Hoagland's nutrient solution. Roots from the plants were sampled after a four-week growth period to visualize and quantify the survival of the recombinant *gfp*-tagged cultures. The plants were replanted after the root sampling and watered in the previous manner.

Transfer Experiment. Poplar trees were inoculated with recombinant *gfp*-tagged microorganisms as described above and planted with different uninoculated plant species to determine if the GEMs will transfer and colonize other plant species, wheat and alfalfa. Both plants were propagated from seeds. The bacterial strain used was Pb5*gfp*2-2 and was prepared for inoculation in the same manner as the previous survival experiment. The plants were grown in 12-inch (0.3 m) diameter containers that were five inches tall. The containers held sterile potting soil at a one-inch depth. The potting soil was autoclaved for one hour. Each poplar was planted with two wheat or two alfalfa plants (Figure 1). Triplicates of each combination were prepared. A single plant of each species (poplar, alfalfa and wheat) was planted as controls. All of the plants were watered under the same conditions as the plants in the survival experiment. The plants were grown for 159 days and then sampled by root sampling.

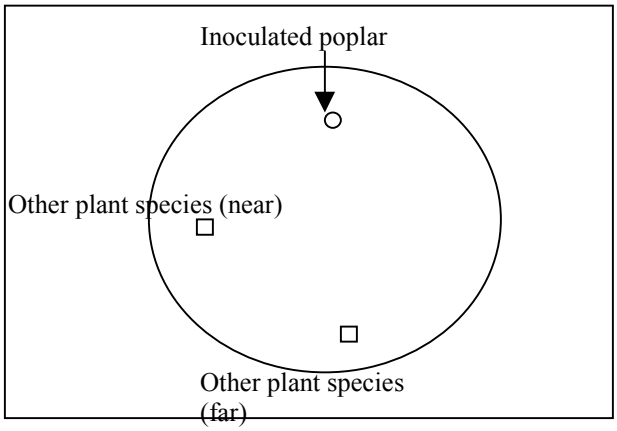

FIGURE 1. Layout of Plants Within Containers

Sampling and Detection Methods. Root sampling was performed in both the survival and transfer experiments. Sampling was done in the same way for both except in the survival experiment plants were replanted following root extraction. To extract and sample the roots the soil was loosed around the roots of the plant and one-inch sections of root were removed from the plants. The root sections were placed in sterile 15-ml screw cap test tubes and suspended in 0.1 M sterile sodium phosphate buffer. If not used immediately for *gfp*-tagged microorganism detection, the roots were refrigerated at 4°C. Three methods were used to detect the GEMs in the previously described experiments; epifluorescent microscopy, most probable number plate counting (MPN) and direct detection. For epifluorescent microscopy, the microorganisms were dislodged from the

one-inch sections of root suspended in buffer solution by vortexing. Then 10 µl samples of the cells in solution were placed on slides and observed using a specific GFP filter set. Unvortexed roots were also removed from the buffer, placed on a slide and directly observed under the microscope. MPN plate counting was performed using one-inch root sections vortexed to suspend the microorganisms. Next, serial dilutions of the solution were completed and then plated onto LB media amended with kanamycin (50 µg/mL) and cyclohexamide (100 µg/mL; to control fungi). Direct detection was performed using tryptic soy broth (TSB) amended with kanamycin (50 µg/mL) and cyclohexamide (100 µg/mL). Root sections were embedded into the media and incubated overnight at 30 degrees Celsius. The plates were then examined for growth of *gfp*-tagged microorganisms using a hand held ultraviolet (UV) light in a dark room.

RESULTS AND DISCUSSION

Growth rates. The growth rates were determined for the recombinant *gfp*-tagged microorganisms (Table 1). A comparison of the determined growth rates to the growth rates of the host strains show that there is minimal difference. The comparison in growth rates demonstrates that the GEMs retain similar cellular activity and growth characteristics of the original host microbes, and provides evidence that the recombinant microorganisms retained the competitive advantage of the host root-colonizing microbes.

TABLE 1. Recombinant Strains and Growth Rates

Host microorganism	Host microorganism growth rate (hr^{-1}) (Shim et al)	Recombinant microorganism	Recombinant microorganism growth rate (hr^{-1})
Pb1	0.853	Pb1gfp2-1	1.087
Pb2	1.024	Pb2gfp2-2	0.979
Pb3	1.179	Pb3gfp2-1	0.747
		Pb3gfp2-3	1.053
Pb5	1.006	Pb5gfp2-2	0.640
Rhizobium 35645	0.593	35645gfp2-1	0.732
		35645gfp2-2	0.993
		35645gfp2-3	0.757

Survival. The *gfp*-tagged bacteria strains were reintroduced into the rhizosphere and monitored. The poplar trees planted with *Rhizobium* 35645*gfp*2-1 did not provide conclusive results, but the plants planted with Pb5*gfp*2-2 showed survival for approximately 28 days under laboratory conditions. Roots extracted in the fourth week had 2 x 10^4 to 26 x 10^4 colonies per inch. The values were determined by MPN plate counting. Epifluorescent microscopy and embedding roots into TSB media were used to visualize fluorescence of bacteria. Analysis in the weeks following replanting (6^{th} and 8^{th}) provided no substantial colony counts.

Transfer. Poplar trees inoculated with recombinant bacteria were planted with other plant species to determine if the recombinant bacteria would transfer and colonize the alternate plant roots. After the long growth period, the root mass was too extensive and intertwined to conclusively differentiate poplar roots and the alfalfa roots, therefore the

results of transfer from sampling the roots was somewhat inconclusive. Sampling of roots known to be poplar revealed growth of a culture that fluoresces at the GFP wavelengths. After observation under the epifluorescent microscope the isolated strain is thought to be original inoculated GEM. Further molecular tools need to be utilized to fully confirm the identification of the observed strain. No roots that were confirmed as alfalfa or wheat revealed GFP activity. However, transfer cannot be fully ruled out due to the ambiguous identity of root tissues.

Detection. The three detection methods were useful in identifying *gfp*-tagged microorganisms. There is potential for photography of the bacteria under the epifluorescent microscope. This is important because through photography the areas of GEM colonization can be visually documented. All of the methods have potential for refining to make visualization and enumeration of the bacteria enhanced.

CONCLUSION

The recombinant bacteria are shown to be stable and to maintain the growth characteristics of the original host bacteria. The GEMs have also demonstrated growth and proliferation when reintroduced into the poplar rhizosphere for an extended period of time. There is no evidence of microorganism transfer and colonization of other plants although this is not fully conclusive. The lack of transfer to and colonization of other plants is evidence that the GEMs will stay contained in the poplar rhizosphere. The results from these experiments give more insight on the interactions between plants and GEMs in a phytoremediation system.

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REFERENCES

Anderson, T. A. and B. T. Walton. 1995. “Comparative Fate of ^{14}C Trichloroethylene in the Root Zone of Plants from a Former Solvent Disposal Site.” *Environ. Toxicol. Chem.* 14(12): 2041-2047.

Bloemberg, G. V., G. A. O'Toole, B. J. J. Lugtenberg and R. Kolter. 1997. “Green Fluorescent Protein as a Marker for *Pseudomonas* spp.” *Appl. Environ. Microbiol.* 63(11): 4543-4551.

Burken, J. G. and J. L. Schnoor. 1996. “Phytoremediation: Plant Uptake of Atrazine and the Role of Root Exudates.” *ASCE J. Environ. Eng.* 122(11): 958-963.

Crowley, D. E., M. V. Brennerova, C. Irwin, V. Brenner and D. D. Focht. 1996. “Rhizosphere Effects on Biodegradation of 2,5-dichlorobenzoate by a Bioluminescent strain of Root-colonizing *Pseudomonas fluorescens*.” *FEMS Microbiol. Ecolo.* 20: 79-89.

Gilbertson, A. G., J. G. Burken, M. W. Fitch, and T. K. Wood. 2000. "Recombinant *gfp*-tagged Root-Colonizing Microbes: Implications on Phytoremediation." In: Leeson,

A., Mager, V.S., Eds, Proceedings of the 6th International Symposium of In Situ and On-Site Bioremediation. Battelle Press.

Normander, B., N. B. Hendriksen and O. Nybroe. 1999. "Green Fluorescent Protein Marked *Pseudomonas fluorescens*:Localization, Viability and Activity in the Natural Barley Rhizosphere." *Appl. Environ. Microbiol.* 65(10): 4646-4651.

Schnoor, J. L. 1997. Phytoremediation. Pittsburgh, PA, National Environmental Technology Applications Center.

Shim, H., S. Chauhan, D. Ryoo, K. Bowers, S. M. Thomas, K. A. Canada, J. G. Burken, and T.K. Wood. 2000. "Rhizosphere Competitiveness of Trichloroethylene-Degrading, Poplar-Colonizing Recombinants." *Applied and Environmental Microbiology* 66: 4673-4678.

Siciliano, S. and J. Germida. 1998a. "Degradation of Chlorinated Benzoic Acid Mixtured by Plant-Bacteria Associations." *Environ. Toxicol. Chem*. 17(4): 728-733.

Siciliano, S. and J. Germida.. 1998b. "Biolog Analysis and Fatty Acid Methyl Ester Profiles Indicate That Pseudomonad Inoculants That Promote Phytoremediation at the Root-Associated Microbial Community of *Bromus biebersteinii*." *Soil Biol. Biochem*. 30(13): 1717-1723.

Tombolini, R., D. J. van der Gaag, B. Gerhardson and J. K. Jansson. 1999. "Colonization Pattern of the Biocontrol Strain*Pseudomonas chloroaphis*MA 342 in Barley Seeds Visualized by using Green Fluorescent Protein (GFP)." *Appl. Environ. Microbiol*. 65(8): 3674-3680.

Yee, D. C., J. A. Maynard and T. K. Wood. 1998. "Rhizoremediation of Trichloroethylene by a Recombinant, Root-Colonizing *Pseudomonas fluorescens* Strain expressing Toluene *ortho*-Monooxygenase Constitutively." *Appl. Environ. Microbiol*. 64(1): 112-118.