VALIDATION OF A MICROCOSM DESIGNED FOR PRE-RELEASE RISK ASSESSMENT OF SOIL MICROORGANISMS USING PLANT GROWTH PROMOTING RHIZOBACTERIA

by

JAMES D. NAIRN

B.Sc., The University of Waterloo, 1989
B.Sc. (Agr), The University of Guelph, 1994

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Department of Forest Science

The University of British Columbia
Vancouver, Canada

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ABSTRACT

Soil microcosms may prove to be a useful tool in the development of standardized methods for evaluating survival and persistence of soil organisms, particularly genetically modified microorganisms, before field release. In this study, a relatively simple soil microcosm, previously shown to be useful for predicting survival of a genetically modified pseudomonad in bulk soil and the rhizosphere of wheat, was tested using two plant growth promoting rhizobacteria (PGPR) strains which were naturally resistant to antibiotics, and spruce seedlings as the test plant. *Bacillus polymyxa* Pw-2R and *Pseudomonas chloroaphis* Sw5-RN were each inoculated onto bare soil and into the rhizosphere of spruce seedlings in field plots as well as in intact soil core microcosms that were incubated under controlled environmental conditions. Survival data collected over a two-year period were used to generate polynomial regressions that modeled the persistence of these strains in the field as well as in the soil microcosms. Comparison of the slopes and intercepts of these regressions indicated that the intact soil core microcosm closely predicted the survival of both PGPR strains in bulk soil and in the spruce rhizosphere. These results demonstrate that this small and inexpensive intact soil core microcosm may be appropriate for general use in assessing field persistence of diverse soil microorganisms before environmental release.

During this study, a temporary loss of antibiotic resistance was observed in both PGPR strains, as they failed to grow on primary isolation agar media with antibiotics. However, they thrived on agar media with antibiotics if they were first

isolated on agar without antibiotics. These results suggest that when using antibiotic resistance as a method to monitor rhizosphere microorganisms, the apparent masking of antibiotic resistance should be evaluated thoroughly.

Bacillus polymyxa Pw-2R and *Pseudomonas chloroaphis* Sw5-RN are both plant growth promoting rhizobacteria. Pw-2R has previously been shown to be capable of colonizing internal root and shoot tissues of hybrid spruce. There were no endophytic Pw-2R detected when attempts were made to isolate Pw-2R from internal tissue in this study. Results further indicated spruce seedling growth was not significantly enhanced by the inoculation with the PGPR strains. These results are consistent with the theory that positive results from PGPR seem to be linked with harsh growing sites and interaction with indigenous microorganisms from the primary site of PGPR isolation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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SECTION 1.
Introduction:

Humankind has utilized naturally occurring microorganisms for the last few decades, for various purposes including the manufacture of human insulin, protein hormones to increase milk production, and enzyme detergents. With the application of molecular biology it is now possible to rapidly develop genetically engineered microorganisms, (GEMs), designed specifically for use in food chemistry, agriculture, wastewater treatment, pharmaceuticals, and mining. Microorganisms that are genetically engineered to carry out specific functions in an unconfined environment need to be a major focus of environmental management. Recently it was stated that there was some urgency in dealing with the concerns of biotechnology because GEMs were likely to be used commercially before the end of the last decade (Levin and Israeli 1996).

Introduction of GEMs into complex, natural ecosystems requires knowledge of the impact they may have upon many different aspects of the ecosystem. Halvorson (1985) suggested such knowledge should include the persistence of the introduced organism, reproduction and survival rates, transport through soil, water and air, frequency of gene transfer to indigenous organisms and their effects on the processes and ultimately the stability of the ecosystems to which they are introduced.

Public perception of the risks and benefits of today's biotechnology plays a large part in the development of GEMs. A moderate level of scientific understanding and general environmental concern has made the public uneasy with the idea of altering the genetic makeup of any organism and releasing it into the environment.

Therefore, it is essential that we assess the risks and benefits of any particular GEM, including the effects on flora and fauna, before any environmental release is attempted. Such initial assessments need to be performed in a controlled setting.

The term "microcosm" is defined as a controlled, reproducible laboratory system designed to simulate a portion of the natural environment (Walter et al. 1991). Terrestrial microcosms incubated in the laboratory are potential tools for evaluating the fate and effects of GEMs to be released into the environment (Bolton et al. 1991). Microcosm studies can help determine whether any genetic modifications to the microorganism are likely to create unacceptable risks, before it is released into the environment (Krimsky et al. 1995). In addition, microcosms provide the researcher with the opportunity to simulate an ecosystem in the laboratory. The small size of the microcosm permits inexpensive replication and management of many factors that are normally present in the field. Even in the simplest soil microcosm, attention must be given to the type of soil used, soil moisture, pH, organic matter content, presence of toxic pollutants, agrochemicals and/or metal ions, species of organism, cycles of freezing/thawing, light/dark cycles, aerobic/anaerobic sites, incubation and wetting/drying cycles (Trevors 1988). Trevors (1988) suggested that not withstanding these complexities, the development of standardized methods for evaluating GEMs would involve microcosms.

Terrestrial laboratory microcosms have been constructed in order to bridge the gap between field soil conditions and laboratory soil systems. The majority of microcosm work has focussed on organisms that have relatively short generation times, facilitating the study of community organization and persistence (Drake et al.

1996). The introduction of GEMs to soil has been reported in many studies using microcosms (Armstrong et al. 1987, Bentjen et al. 1989, Fredrickson et al. 1989, Fredrickson et al. 1990, Bolton et al. 1991, Walter et al. 1991, Angle et al. 1995). The fate of GEMs transported through the soil in microcosms has also been studied (Bentjen et al. 1989, Trevors et al. 1990). Soil microcosms appear to be a valuable tool for studying microorganisms in natural soil environments (Bolton et al. 1991, Drake et al. 1996, Hill and Top 1998, Carpenter 1996, Teuben and Verhoef 1992).

Standardization must occur in order to facilitate comparisons between different sites. In spite of the continued discussion of the reliability of microcosm systems, there has been little effort to define some form of standardization (Verhoef 1996). A universal microcosm design may be used as an initial step in studying the increasing levels of complexity in the soil ecosystem. A standard microcosm may allow controlled comparisons of less complex systems after which increasing dynamics may be introduced to create a more elaborate system. A universal standard microcosm should also be simple to use and be relatively inexpensive.

A microcosm has recently been developed at the University of Maryland for testing the survival and persistence of recombinant bacteria (GEMs) released into the environment (outdoor field plots). The microcosm is an intact soil core 18 cm x 5 cm. Previous work demonstrated that the intact soil core closely predicted the survival of a pseudomonad in the field (Angle et al. 1995). To further validate this work, identical microcosms were tested concurrently at several locations across North America. These were at the University of Maryland, Carleton University, the University of Saskatchewan, Agriculture and Agri-Food Canada in Ottawa, McGill

University and the University of British Columbia. After a two-year study, it was determined that the microcosm could be used to predict the field survival of *Pseudomonas chloroaphis* 3732RN-L11 (Gagliardi et al. 2000). The chloroaphis strain used in the study had been isolated from the rhizosphere of wheat and had a lacZY gene inserted into the chromosome as a marker (Barry 1986). Although the inoculum density varied with sites, the survival slope was consistent between the microcosm and the field at most sites (Gagliardi et al. 2000). At the conclusion of the study it was decided that further validation of this microcosm should include the use of other microorganisms and other species of plants.

The term plant growth promoting rhizobacteria (PGPR) was first used to describe naturally occurring bacteria that are capable of colonizing the roots of plants and stimulating plant growth (Kloepper and Schroth 1978). PGPR activity has been studied in many strains of soil bacteria (Rovira 1963, Kloepper et al. 1986, Kloepper et al. 1988, Kloepper et al. 1989, O'Neill et al. 1992b, Chanway and Holl 1992, Chanway et al. 1994). Recent work has been completed on the influence of Plant Growth Promoting Rhizobacteria (PGPR) on the survival and growth of outplanted conifer seedlings (Chanway et al. 1997).

PGPR effects on trees have been studied only recently. In some cases, PGPR inoculation of conifer seedlings has resulted in large, statistically significant gains in plant biomass (Chanway 1997). The demands of a rapidly increasing global population necessitate enhancing forest productivity. PGPR inoculum has the potential to increase survival and growth of transplanted seedlings in an inexpensive and environmentally benign manner.

Bacteria that colonize internal root tissues have been described as root endophytes (Kloepper et al. 1992). Root endophytes may be more effective as PGPR than rhizosphere bacteria because they are more likely buffered against any environmental extremes due to the location inside the root tissue (Shishido et al. 1995, Chanway et al. 1994). Root endophytes are found primarily intercellularly in both cortical and vascular root tissues (Kloepper et al. 1992). Establishment of these endophytic populations, which act to stimulate conifer seedling growth, may result in greater inoculum persistence if the microorganisms are established within the conifer root tissues before the seedlings are outplanted. Eventual displacement of the endophytic inoculum by indigenous species would not detract from the beneficial effects of the inoculum because the benefits of the PGPR appear to be achieved within the first few years after outplanting (Chanway 1997). Endophytic PGPR have been shown to promote growth in spruce outplantings (Chanway et al. 1994), pine greenhouse trials (Shishido et al. 1995) and field trials (Chanway and Holl 1994), and short-term effectiveness with lodgepole pine and hybrid spruce seedlings at certain reforestation sites (Chanway et al. 1997).

Work with PGPR has recently been performed on one year old, container grown, lodgepole pine and spruce seedlings which were inoculated with antibiotic resistant derivatives of three *Bacillus* strains (Pw2, L6 and S20) and three *Pseudomonas* strains (Sw5, Sm3 and Ss2) (Chanway et al. 1997). This study confirmed the short-term benefits of PGPR for conifer seedlings and indicated that the positive effects of a single inoculation at planting can extend at least through the second year of growth.

The focus of the work presented in this thesis deals with two of these plant growth promoting isolates, *Bacillus polymyxa* strain Pw-2R as well as *Pseudomonas fluorescens* strain Sw5-RN. Pw-2R was isolated from within surface sterilized roots of a lodgepole pine seedling and is a seedling growth promoting derivative of the parental PGPR strain and is naturally resistant to an elevated rifamycin concentration (Shishido et al. 1996a). Sw5-RN is also a growth promoting derivative of parental PGPR strain Sw5, and is naturally resistant to elevated rifamycin and nalidixic acid levels (Shishido et al. 1996b). Use of antibiotic resistant mutants of parental strains facilitates detection of inoculant bacteria in the presence of indigenous soil microorganisms (Kloepper and Beauchamp 1992).

Recently, the temporary loss of antibiotic resistance, termed 'antibiotic masking', has been observed with isolation of bacterial endophytes from inside cotton plants (McInroy et al. 1996). Antibiotic masking has been described as the temporary loss of the ability to thrive in the presence of specific levels of antibiotics. Replica plating techniques must be employed in order to determine if the target organisms are present and displaying characteristics associated with antibiotic masking. The work described in this study investigated the possibility of antibiotic masking by the target organisms in the rhizosphere of seedlings as well as in internal root and shoot tissues.

This study compared the survival data of the PGPR strains *Bacillus polymyxa* Pw-2R and *Pseudomonas fluorescens* Sw5-RN inoculated onto interior spruce seedlings and bare soil. The inoculation occurred in intact soil core microcosms in a controlled laboratory setting as well as in field plots. These experiments were

undertaken in an attempt to further validate the use of this particular microcosm (Angle et al. 1995) for application as a future standard for risk assessment.

In particular I tested the following hypotheses:

1) *Bacillus polymyxa* strain Pw-2R and *Pseudomonas fluorescens* strain Sw5-RN inoculated onto bare soil would decline in population size similarly in the field and in the intact soil core microcosm to undetectable levels within 10 months. Both population levels were expected to drop below the limit of detection soon after inoculation as both strains were originally found in the rhizosphere. Therefore, a 10 month time span was chosen as it was expected that the populations would reach zero in the first few months.
2) *Bacillus polymyxa* strain Pw-2R and *Pseudomonas fluorescens* strain Sw5-RN inoculated into the rhizosphere of the spruce seedlings would decline in population more gradually than in bare soil, so that the populations would not decline below detectable limits before the end of the trial. There would be a similar rate of decline between the field and intact soil core microcosm.
3) An increase in biomass would occur in the seedlings inoculated with the PGPR strains *Bacillus polymyxa* Pw-2R and *Pseudomonas fluorescens* Sw5-RN in both the field and intact soil core microcosms.
4) *Bacillus polymyxa* Pw-2R would be found in internal root tissue of the inoculated seedlings in both the field and the intact soil core microcosms.

SECTION 2.
Materials and Methods:

2.1 Microorganisms:

Two bacterial strains previously shown to stimulate conifer seedling growth were used for both growth promotion and microcosm analysis. Both strains originated from the Williams Lake area in British Columbia, Canada. *Bacillus polymyxa* strain Pw-2R is a rifamycin-resistant derivative of *Bacillus polymyxa* strain Pw-2 which was originally isolated from within surface sterilized roots of a naturally regenerating lodgepole pine seedling (<3 years old) (Shishido et al. 1995). Pw-2R is naturally resistant to an elevated (200 $mg \cdot L^{-1}$) rifamycin concentration (Shishido et al. 1996a). *Pseudomonas fluorescens* strain Sw5-RN is a rifamycin- and nalidixic acid-resistant derivative of *Pseudomonas fluorescens* strain Sw5 which was isolated from the rhizosphere of a hybrid spruce (*Picea glauca x engelmannii*) seedling and is naturally resistant to elevated (100 $mg \cdot L^{-1}$) rifamycin and nalidixic acid levels (Shishido et al. 1996b). The antibiotic-resistant strains are similar to their parental wild-type strains with respect to colony morphology, 23S-rDNA sequences, Biolog carbon substrate utilization, and intrinsic antibiotic resistance to streptomycin, kanamycin and tetracycline (Shishido et al. 1996a, b).

Bacillus polymyxa strain Pw-2R as well as *Pseudomonas fluorescens* strain Sw5-RN were maintained in 50% strength tryptic soy broth (TSB) with 20% glycerol at -80°C. The two strains were cultured on 50% strength tryptic soy agar (TSA) amended with 200 $mg \cdot L^{-1}$ rifamycin for Pw-2R and 100 $mg \cdot L^{-1}$ nalidixic acid and rifamycin for Sw5-RN. Cycloheximide (100 $mg \cdot L^{-1}$) and nystatin (50 $mg \cdot L^{-1}$) were added to all TSA plates to inhibit fungal growth. Inoculum was prepared by placing a

single colony of each strain isolated from their respective TSA plates into separate 10 mL aliquots of TSB. The *B. polymyxa* Pw-2R inoculum broth contained 200 $mg \cdot L^{-1}$ rifamycin while the *P. fluorescens* Sw5-RN inoculum broth contained 100 $mg \cdot L^{-1}$ nalidixic acid and rifamycin. Both media contained 100 $mg \cdot L^{-1}$ cycloheximide and 50 $mg \cdot L^{-1}$ nystatin for their antifungal abilities. The cultures were shaken on a rotary shaker at 175 RPM for 12h and 15h, for Pw-2R and Sw5-RN respectively, to an approximate density of 10^8 colony forming units $(CFU) \cdot mL^{-1}$. Following this, 1.0 mL from each was placed in multiple flasks containing 1.0 L of fresh 50% strength tryptic soy broth amended with the antibiotics and antifungals previously described. The broth was shaken on a rotary shaker at 175 RPM to an approximate density of 10^8 $CFU \cdot mL^{-1}$. The resulting cultures were centrifuged at 4°C and 5000 x g for 30 minutes; cells were then resuspended in the same volume of 10 mM sterile phosphate buffer (SPB) to an approximate density of 10^8 $CFU \cdot mL^{-1}$.

2.2 Seedlings:

One year old, container grown spruce was used for these trials. The seedlings, (*Picea glauca* (Moench) Voss X *Picea engelmannii* Parry), were grown in 415B styroblocks in a greenhouse at Pelton Reforestation Ltd., the year prior to this trial. The seedlings were placed at -18°C at the beginning of November 1997, then removed at the end of April 1998, four days prior to planting. The seed originally came from the Interior Coastal Western hemlock zone (ICH) in the Mount Robson Seed Planning Zone in British Columbia, Canada. The seed was collected in 1990

from an elevation of 1150 metres at a mean latitude of 52 degrees and a mean longitude of 118 degrees.

2.3 Field Plot Design and Planting:

Government of Canada approval was required and obtained for the release of *Bacillus polymyxa* strain Pw-2R as well as *Pseudomonas fluorescens* strain Sw5-RN into the field. Approval was obtained by submission of form T-4-103 to the Canadian Food Inspection Agency. The field plot area to be used was covered with black polyvinyl sheeting for two months in an attempt to kill any existing vegetation on the site. Seven-metre square and two-metre square plots were established adjacent to a Douglas-fir stand in the Coastal Western Hemlock biogeoclimatic zone on the University of British Columbia campus. The land had been previously cleared and tilled into soil resembling an agricultural soil. Each treatment (Pw-2R and Sw5-RN) had four replicate plots for both seedlings and bare soil. Plot layout and design were completely randomized. Each plot containing seedlings consisted of 36 trees in a 6 X 6 arrangement. Each plot was 7 X 7 metres and each seedling was spaced 1 metre from the adjacent seedlings. The plots were laid out on level ground in a double row of four with a 1 metre spacing separating each plot. Six seedlings chosen randomly in each plot were not inoculated and were left as controls. Similarly, two-metre square plots were established for bare soil trials and were also completely randomized. Corrugated plastic edging 15 cm high was buried 7.5 cm deep around each plot to prevent losses of inoculum due to runoff and to inhibit root interactions between adjacent plots. All surface organic debris was removed from

the surface inside each field plot by hand. To maintain similar soil conditions between microcosms and the field, adjacent plots with the surface soil prepared identically were used for the collection of the microcosms. For the duration of the trial, plots were weeded by hand. When necessary, weeds removed from the soil were kept within the plot to prevent contamination of adjacent plots. Watering occurred only when drought conditions existed (more than 14 days without rain).

2.4 Microcosms:

Microcosms were constructed of 5 cm inner diameter schedule 40 polyvinyl chloride (PVC) plumbing pipe, 17.5 cm long, beveled to 45° on one outside edge. The cores, beveled edge down, were driven into the ground and gently removed by hand. A plastic hammer with a 15 cm wide head filled with 100 grams of loose ballast (McMaster-Carr Co., New Brunswick, New Jersey) was used to drive the microcosms 15 cm into the soil. The microcosms were spaced approximately 2 cm apart and were randomly collected from different sites adjacent to the established plots. Microcosms were removed from the soil by excavating on one side to dig a trench along each line of cores driven into the ground. A small shovel was used to break the cores from the soil profile and to create a uniform bottom surface. Two layers of aluminum foil held in place with elastic bands were placed over the bottom of each core after the microcosms had been cleaned externally of any adhering soil. Following core extraction from the field, 345 cores were planted each with one spruce seedling and 200 cores were left as bare soil. Cores were immediately returned to the laboratory and placed in the greenhouse. Ten soil core microcosms

were weighed prior to harvesting the remainder in order to determine the weight of an intact soil core microcosm at 60% saturation. Microcosms were weighed and watered to 75% saturation immediately prior to inoculation. Each microcosm was inoculated with 5 mL of culture, followed by 10 mL of sterile distilled water. This resulted in each core being at approximately 90% saturation after inoculation.

Microcosms were weighed on an electronic balance and watered when necessary in order to maintain 60% saturation for the duration of the trial. The microcosms were inoculated within 24 hours.

2.5 Inoculation:

Microcosms without seedlings (bare soil) were inoculated by dripping 5 mL of the cultures evenly onto the soil surface. Following inoculation, each microcosm received an additional 10 mL of sterile, distilled water dribbled evenly onto the soil surface. Each bare soil field plot contained an inner area (2m X 2m) of approximately 1000 cores, therefore, 5 L of inoculum was slowly spread over the plot using a sterilized polyethylene gravity-fed watering bottle (ECO-SPOUT, M.S. Harman Co., Columbus, OH) for even distribution. This was followed by application of 10 L of sterile distilled water spread in the same manner. Seedlings in the field plots and rhizosphere microcosms were inoculated by applying 5 mL of culture to the centre of the plug, approximately 7 cm from the top, using a 10cc syringe and 18 gauge needle. Microcosms were watered throughout the experiment to maintain 60% saturation as described previously. After each watering the cores were randomly relocated in the greenhouse space.

2.6 Sample Collection:

The sampling schedule occurred over a period of 17 months. Bare soil and rhizospheres from both field and laboratory microcosms were assayed for bacterial colonization following a previously established protocol (Angle et al. 1995). Briefly, Angle (1995) inoculated the recombinant organism *Pseudomonas aureofaciens* RN-L11 into intact soil core microcosms and field plots containing bare soil or wheat plants. Survival data from the microcosms was compared with data obtained from the field release.

2.6.1 Field Sample Collection:

Field samples were obtained on the day of sampling immediately prior to extraction in the lab. Bare soil plots were sampled using a stainless steel T-handle Oakfield soil corer. Four 2.5 cm diameter cores were randomly taken from the top 15 cm of each bare soil plot. Each hole created by the coring device in each plot was marked to avoid resampling a previous core. The cores from each plot were pooled into a sterile plastic bag and transported in a cooler to the laboratory.

For the collection of rhizosphere samples, a sterile shovel was used to dig out the entire root system in a 30 cm diameter around each individual tree to an approximate depth of 15 cm. One tree was randomly selected from each plot on each sampling day. Three control rhizosphere samples were taken periodically from random plots. Loosely adhering soil was removed and the rhizosphere samples along with the attached plant were placed in a sterile plastic bag and transported in a cooler to the laboratory. Instruments used for sampling were flame-sterilized with 70% ethanol initially and following that, after each plot had been sampled.

2.6.2 Microcosm Sample Collection:

Microcosms were not watered for 48 hours prior to sampling to ensure soil or roots would not adhere to the core or aluminum foil attached to the bottom of the microcosm. Samples for bare soil and rhizosphere microcosms containing strains Pw-2R and Sw5-RN as well as controls were randomly chosen from the greenhouse immediately prior to extraction in the lab. Four samples of each Pw-2R and Sw5-RN for both bare soil and rhizosphere were harvested each sampling day. Three control samples were harvested on selected sampling days (days 0, 52, 66, 94, 165, 375, 389, 403, 445, 489 and 509). Soil, and rhizospheres in microcosms, were removed in the same way to prevent compaction and undue disturbance of the contents. The microcosms were held firmly, upside down, over a 4-mm sieve. The top of the core (facing down) was tapped with the plastic hammer (previously mentioned) until the soil core fell out into the sieve. This procedure was the same for bare soil and rhizosphere cores. Once the rhizosphere samples were removed from the cores, the root systems were removed from the shoots at the root crown. The fresh weight of the shoots were recorded and shoots were placed in paper bags in a drying oven set at 75°C for a minimum of 3 days and each weight recorded.

2.7 Soil Sample Processing:

For both field and microcosm samples, the soil was thoroughly sieved through a 4-mm screen to remove organic and inorganic debris. Each resulting fresh soil sample was weighed and then well mixed with a sterile spatula. A sub-sample of 10-g was aseptically removed for the extraction of bacteria. The 10-g sub-sample was

added to 95 mL of 10 mM sterile phosphate buffer (SPB) and placed in a sterile glass blender (Waring Corp, New Hartford, CT). Each sample was blended for 1-minute followed by an additional 1-minute period to allow the soil to settle in the blender. Serial dilutions were made in 9 mL of 10 mM SPB using 1 mL of the original solution pipetted from the blender. The samples were drawn from the middle of the solution in the blender.

2.8 Rhizosphere Sample Processing:

For both field and microcosm rhizosphere samples, the root systems were extracted onto the sieve. The loosely adhering soil, organic and inorganic matter was removed with the pressure of a sterile spatula. The entire rhizosphere with tightly adhering soil was weighed and recorded as fresh weight. The entire rhizosphere was then placed in 90 mL of 10 mM sterile phosphate buffer (SPB). The sample was shaken for 1 minute on a rotary shaker at 1000 RPM. Soil was allowed to settle from the solution for 1 minute. Serial dilutions were made in 9 mL of sterile SPB using 1 mL pipetted from the original solution. The samples were drawn from the middle of the solution container. Roots were then removed from the dilution bottles and thoroughly washed to remove any remaining soil. The roots were weighed, dried at 75°C for a minimum of 3 days and each weight subsequently recorded.

2.9 Sample Plating and Enumeration:

The initial bare soil sampling occurred on the first day after inoculation. The initial rhizosphere sampling did not occur until 10 days after inoculation. Sampling of the bare soil continued until two consecutive sampling days resulted in no target organisms above the limit of detection. Sampling of the rhizospheres occurred throughout the entire experiment. The minimum level of detection was 10 colony forming units (CFU)·g^{-1} of soil and 1.5 CFU·g^{-1} of fresh root weight for the heaviest rhizosphere samples. All rhizosphere samples were initially diluted in 90 mL of 10 mM SPB, therefore the limit of detection varied with the weight of fresh root biomass. The limit of detection for the rhizospheres was derived from the equation [(plate count mean) x (Dilution Factor) x (Volume / Rhizosphere Weight)], therefore the lowest limit was found with the highest rhizosphere weight. Tryptic soy agar (50% strength) plates were amended with 200 mg·L^{-1} rifamycin and 100 mg·L^{-1} cycloheximide for Pw-2R and 100 mg·L^{-1} nalidixic acid, rifamycin and cycloheximide for Sw5-RN. All TSA plates also contained 50 mg·L^{-1} of nystatin. Plates were spread with 100 μL of sample from the appropriate serial dilutions. When the population levels reached the limit of detection, plates from the lowest dilution were spread with 1 mL from the original sample. Each dilution from each sample was plated in triplicate. The plates were allowed to dry for 1 hour before they were inverted and placed in the dark at 20°C. Incubation continued until target organisms were visible or until the plates became too contaminated to read. Plates from dilutions with 30 to 300 target colonies were counted. Lower counts from the lowest dilutions were used near the end of sampling, as they were the only measurement available.

2.10 Sample Plating to Uncover Antibiotic Resistance:

During the second growing season (day 354 to day 489) all samples were checked for the masking of antibiotic resistance (McInroy et al. 1996). Plates used for the initial plating of the samples from the serial dilutions contained cycloheximide (100 $mg \cdot L^{-1}$) and nystatin (50 $mg \cdot L^{-1}$) in 50% strength tryptic soy agar (TSA). After aliquots had been spread on the agar, plates were allowed to dry for 1 hour before they were inverted and placed in the dark at 20°C. Plates were left for 24 to 48 hours and any suspected target organisms were then counted. Each plate from each sample was then replicated using a Replica Plating Tool (VWR Canlab, West Chester, PA). The plates were replicated onto 50% strength TSA amended with 200 $mg \cdot L^{-1}$ rifamycin and 100 $mg \cdot L^{-1}$ cycloheximide for Pw-2R and 100 $mg \cdot L^{-1}$ nalidixic acid, rifamycin and cycloheximide for Sw5-RN. All TSA plates also contained 50 $mg \cdot L^{-1}$ of nystatin. Following replication, plates were inverted and incubated in the dark at 20°C. Plates from dilutions with 30 to 300 target colonies were counted. Lower counts from the lowest dilutions were used near the end of sampling, as they were the only measurement available.

2.11 Sampling for Internal Plant Colonization:

After processing the rhizosphere sample described previously, Pw-2R root systems were removed from the dilution bottles and thoroughly washed. A sample selection from random areas of a root system was taken and surface sterilized by immersion in 2.5% NaClO for 2.5 minutes followed by three 30 second rinses in sterile 10 mM sterile phosphate buffer (SPB). Shoot tissues were taken from the

same seedling as the roots. A sterile scalpel was used to remove the bark and a second sterile scalpel was used to remove a sample from the shoot. Tissues were then aseptically triturated with a mortar and pestle in enough SPB to result in a minimum level of detection of 100 CFU g^{-1} of fresh root (i.e. 0.100g tissue in 1 mL SPB). The resulting homogenate was serially diluted and 0.100-mL aliquots were plated onto 50% strength tryptic soy agar (TSA) plates amended with 100 $mg \cdot L^{-1}$ cycloheximide and 50 $mg \cdot L^{-1}$ nystatin. The effectiveness of the surface sterilization was verified by imprinting the sterilized roots on the same TSA plates described above. Plates were allowed to dry for 1 hour before they were inverted and placed in the dark at 20°C. Plates were left to incubate for 24 to 48 hours. Any samples that showed target organism (Pw-2R) growth on the imprinted plates were removed at this point as they indicated inefficient surface sterilization which would lead to erroneous internal root colonization counts. Other samples that did not show target organism growth on the imprint plates were counted for suspected target organisms. In the second year of sampling, (day 333 to day 489), each plate from each sample was replicated using a Replica Plating Tool (VWR Canlab, West Chester, PA). The plates were replicated onto 50% strength TSA amended with 200 $mg \cdot L^{-1}$ rifamycin, 100 $mg \cdot L^{-1}$ cycloheximide and 50 $mg \cdot L^{-1}$ nystatin. Following replication, plates were inverted and placed in the dark at 20°C for 48 to 96 hours. Any visible target organisms were counted.

2.12 Statistical Analysis:

Laboratory and field experiments were conducted using four replicates in a randomized complete block design.

2.12.1 Microcosm Validation:

All plate count data were transformed (log(10) +1) prior to statistical analysis in order to improve homoscedasticity and normal distribution. The minimum detectable population size was 10 colony forming units $CFU \cdot g^{-1}$ of soil and 1.5 $CFU \cdot g^{-1}$ of fresh root weight for the largest rhizospheres. Samples that yielded no growth on dilution plates were assumed to be uncolonized and were given a value of zero. This resulted in a conservative estimation, as true zero population counts were not expected (Kloepper and Beauchamp 1992). Analysis of variance (ANOVA) was conducted for data combined over growing seasons and sampling days to test the slopes of the polynomial regressions of survival rates for significant differences between field plots and intact laboratory soil core microcosms. The ANOVA analyzed the decline in the population size of microorganisms between the laboratory microcosms and field plots over time. Comparison of two slopes was possible using ANOVA with two F-ratio tests (Kozak 1964) and Systat 6.0 statistical software (SPSS Inc., Chicago, IL).

The first F-ratio analysis tests the hypothesis that the two regression equations are describing two parallel surfaces. Two residual sums of squares (SS) must be considered:

1). The residuals for an unrestricted, individual or maximum (max) model in which the total sums of squares are defined as:

$SS_{(max)} = SS_{(Field\ data)} + SS_{(Lab\ data)}$ and degrees of freedom

$DF_{(max)} = DF_{(Field\ data)} + DF_{(Lab\ data)}$.

2). The residuals for the restricted model which forces the regression lines to be parallel (par) in which the total sums of the squares are defined as:

$SS_{(par)} = SS_{(Field\ data + Lab\ data + Dummy)}$

$DF_{(par)} = DF_{(Field\ Data + Lab\ Data + Dummy)}$

The term "Dummy" refers to the variable used to represent the two different regression equations (either 0 or 1). The Dummy variable allows the single regression equation to represent both field and lab data.

The F-ratio test for parallelism was dependent upon the degree of polynomial tested but in each case followed the equation:

$$F_{test} = \frac{\dfrac{SS_{(par)} - SS_{(max)}}{DF_{(par)} - DF_{(max)}}}{\dfrac{SS_{(max)}}{DF_{(max)}}}$$

The second F-ratio analysis tests the hypothesis that there is no separation between the regression lines and that they could be coincidental and replaced by a single regression equation. Two residual sums of squares must be considered:

1). The residuals for the restricted model which forces the regression lines to be parallel (par) in which the sums of the squares:

$SS_{(par)} = SS_{(Field\ data + Lab\ data + Dummy)}$

$DF_{(par)} = DF_{(Field\ Data + Lab\ Data + Dummy)}$

2). The residuals for a single regression surface fitted for the data when all of the data are combined (com):

$$SS_{(com)} = SS_{(Field\ data + Lab\ data)}$$

$$DF_{(com)} = DF_{(Field\ Data + Lab\ Data)}$$

The F-ratio test for coincidence was dependent upon the degree of polynomial tested but in each case followed the equation:

$$F_{test} = \frac{\dfrac{SS_{(com)} - SS_{(par)}}{1}}{\dfrac{SS_{(par)}}{DF_{(par)}}}$$

2.12.2 Plant Growth Promotion:

Analysis of variance was conducted on all data for each sampling day to determine significant differences in plant growth responses between treatments. Data were transformed (log(10) +1) prior to statistical analysis in order to improve homoscedasticity and normal distribution. Total biomass, shoot and root growth responses to PGPR treatment were assessed for significant differences using ANOVA with the F-ratio tests mentioned in the previous section (Kozak 1964) and Fisher's Least Significant Difference mean comparison.

SECTION 3.
Results:

3.1 Evaluation of Experimental Design:

A randomized complete block design was used to evaluate laboratory and field experiments. An analysis of variance was used to test the homogeneity of the field plots. There was no significant difference ($p<0.05$) between any of the field plots for either *Bacillus polymyxa* Pw-2R or *Pseudomonas fluorescens* Sw5-RN inoculations. The microcosm cores were chosen from random locations adjacent to the field plots, watered and arranged randomly each day of watering, and selected for sampling at random.

3.2 Microcosm Evaluation:

3.2.1 Bare Soil:

Testing with *Bacillus polymyxa* Pw-2R and *Pseudomonas fluorescens* Sw5-RN included inoculating the microorganisms onto bare soil in the field plots and onto bare soil in the laboratory microcosms.

Following inoculation the decline in population was rapid for both Pw-2R and Sw5-RN. Colony forming units (CFU) measured the remaining population levels over time.

The initial inoculation density for Pw-2R was approximately 2.0×10^6 colony forming units (CFU)$\cdot mL^{-1}$. Each bare soil laboratory microcosm was inoculated with 5 mL, or approximately 1.0×10^7 CFU. Microcosms contained approximately 300 cm^3 of soil. The average weight of each sample at approximately 60% saturation was 500 grams. Therefore, with an inoculation of 1.0×10^7 CFU per 500 g, the expected

inoculation density of Pw-2R was approximately 2.0 x 10^4 CFU·g^{-1} fresh soil. The composited four core samples taken from the bare soil field plots with the Oakfield sampler also contained approximately 300 cm^3 of soil and weighed approximately 500g. Therefore the expected inoculation density of Pw-2R in the field plots was also approximately 2.0 x 10^4 CFU·g^{-1} fresh soil.

Sampling for survival of Pw-2R in bare soil in both the field plots and laboratory intact soil core microcosms began the day following inoculation. Both field plots and laboratory microcosms were examined each sampling day. A loss in magnitude of up to 1 log was expected after inoculation due to die-off of the organism. Therefore the expected range in capture size of Pw-2R in the field plots and laboratory microcosms was 2.0 x 10^3 to 2.0 x 10^4 CFU·g^{-1} fresh soil. The results of sampling on day 1 after inoculation resulted in Pw-2R population counts ranging between 2.67 x 10^2 and 7.37 x 10^3 CFU·g^{-1} fresh soil for both field plots and laboratory microcosms.

Population counts for *Bacillus polymyxa* Pw-2R in bare soil, in both field plots and laboratory microcosms, declined steeply within the first 60 days after inoculation (Figure 1). The population decline of Pw-2R in the laboratory microcosm displayed the same polynomial trend as the population of Pw-2R in the field plots. The line of best fit for both field and microcosm Pw-2R populations followed a cubic polynomial regression. In both cases the target organism, *Bacillus polymyxa* Pw-2R, was no longer detectable after 56 days (below 10 CFU·g^{-1} fresh soil). An F-ratio test to compare the regression slopes for the Pw-2R inoculated bare soil field plots and laboratory microcosms indicated no significant difference ($p<0.05$) in slopes. The

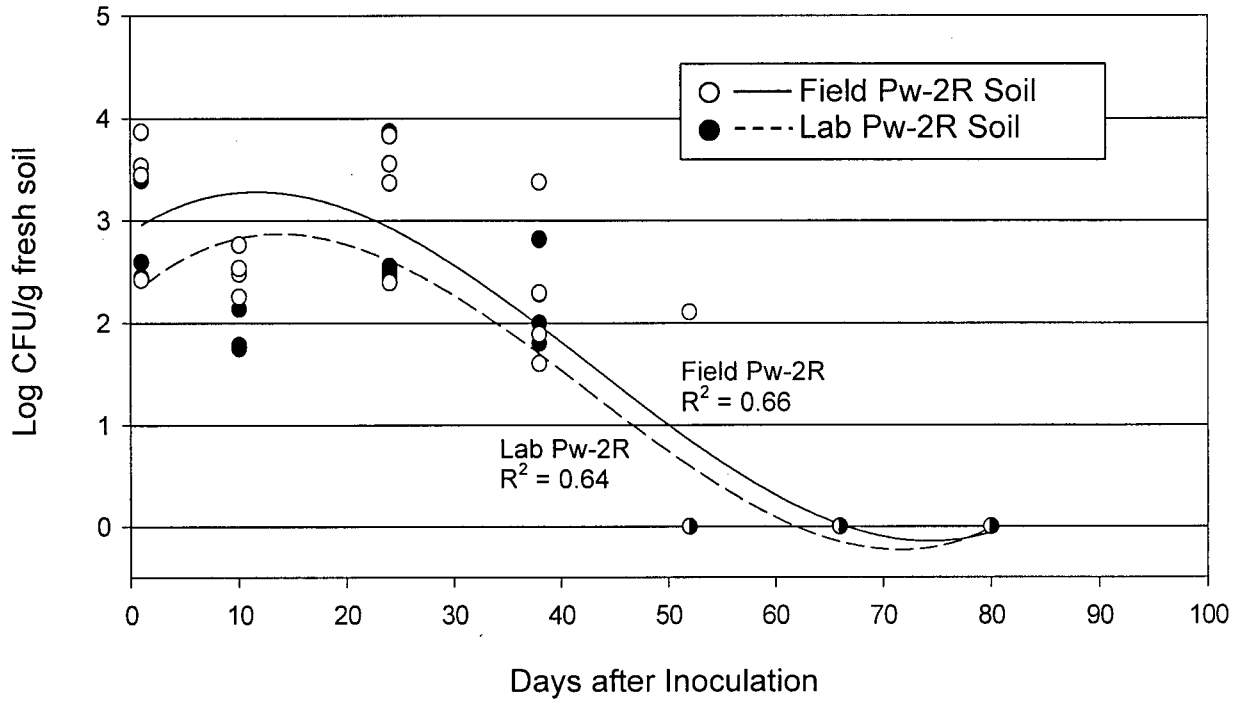

Figure 1. Regression analysis for the survival of *Bacillus polymyxa* (Pw-2R inoculated onto bare soil in both laboratory intact soil core microcosm and field plots. Populations are measured in colony forming units (CFU remaining. Analysis of variance revealed the slopes, and the separatio of the lines, are not significantly different at $p<0.05$. Each circl represents a single sample. ◑ Represents overlapping data points.

regression slopes were therefore parallel (Appendix-Table A1). The results also indicated no significant difference ($p<0.05$) in the separation between the lines, therefore the two regression lines used to predict the survival of the Pw-2R populations were coincidental (Appendix-Table A2).

The trial for the *Pseudomonas fluorescens* Sw5-RN inoculated onto bare soil followed the same protocol as that for the *Bacillus polymyxa* Pw-2R trial. The initial inoculation density for Sw5-RN was approximately 1.0×10^7 colony forming units (CFU)·mL^{-1}. Each bare soil laboratory microcosm was inoculated with 5 mL, therefore each was inoculated with approximately 5.0×10^7 CFU. Microcosms contained approximately 300 cm^3 of soil. The average weight of microcosm core at approximately 60% saturation was 500 grams. Therefore, with an inoculation of 5.0×10^7 CFU per 500 g, and an expected initial die-off up to 1 log in magnitude, the anticipated range in capture size of Sw5-RN in the laboratory microcosms was 1.0×10^4 to 1.0×10^5 CFU·g^{-1} fresh soil. The composited four core samples taken from the bare soil field plots with the Oakfield sampler also contained approximately 300 cm^3 of soil and weighed approximately 500g. Therefore the expected range in capture size of Sw5-RN in the field plots was also 1.0×10^4 to 1.0×10^5 CFU·g^{-1} fresh soil.

The day following inoculation was the first sampling day for Sw5-RN. Both field plots and laboratory microcosms were examined each sampling day. Sampling on day 1 after inoculation resulted in Sw5-RN population counts between 1.04×10^2 and 6.83×10^3 CFU·g^{-1} for both laboratory microcosms and field plots.

The data for the population decline of *Pseudomonas fluorescens* Sw5-RN inoculated onto bare soil in the field and in the laboratory microcosms had to be log

transformed twice. This satisfied the assumptions for analysis of variance that the data have a normal distribution and homoscedasticity. After transforming the data, the line of best fit for both field and microcosm data followed a cubic polynomial regression (Figure 2). The population of Sw5-RN was below the detectable limit of 10 colony forming units (CFU)·g^{-1} fresh soil 56 days after inoculation. An F-ratio test was used to compare the declining regression slopes and indicated parallelism, as there was no significant difference ($p<0.05$) between the slope of the two lines (Appendix-Table A3). Further testing revealed that there was no significant difference ($p<0.05$) in the separation of the lines (Appendix-Table A4). The regression lines for the decline in population of Sw5-RN inoculated onto bare soil in both the field and microcosms were concurrent.

3.2.2 Rhizosphere:

The survival of *Bacillus polymyxa* Pw-2R and *Pseudomonas fluorescens* Sw5-RN inoculated into the rhizospheres of one year old hybrid spruce were different from their survival in bare soil. The survival trends still possessed negative slopes, but the microorganisms tended to survive longer in the rhizosphere. The time to reach the lowest limit of detection was much longer in the rhizospheres (Figures 3 and 4).

Each seedling was inoculated by applying 5mL of culture to the centre of the plug using a 10cc syringe and 18-gauge needle. Therefore, the inoculation density of Pw-2R was approximately 1.0×10^7 colony forming units per rhizosphere. The first rhizosphere sampling occurred 10 days after inoculation. Sampling on day 10 after

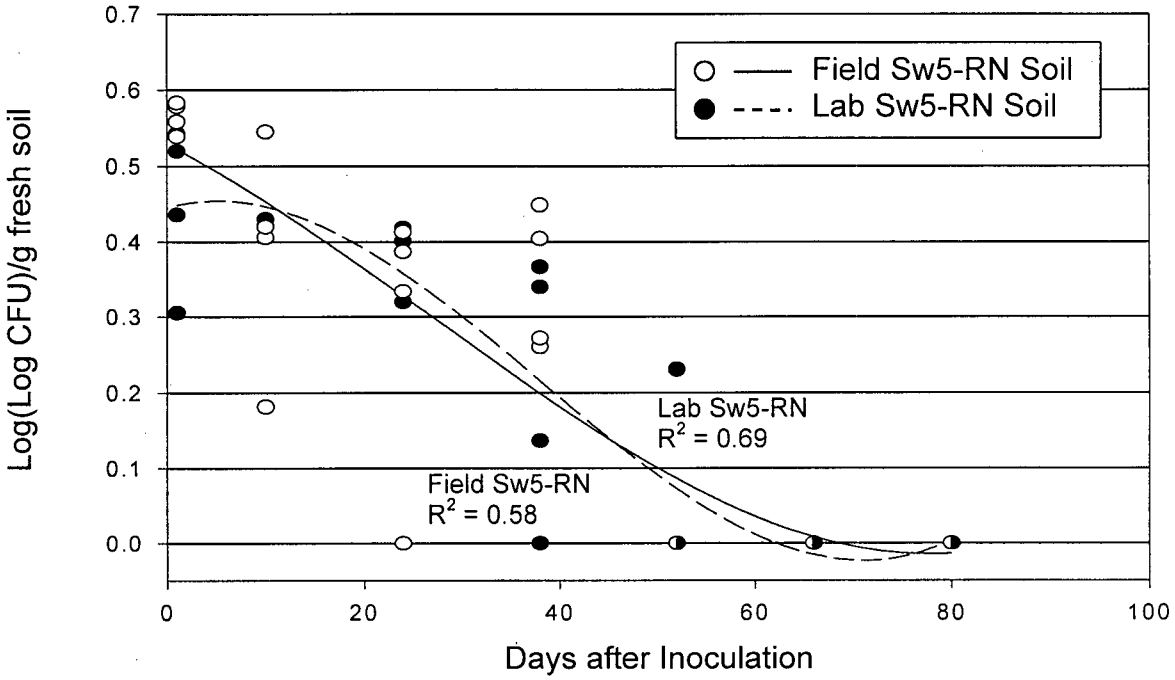

Figure 2. Regression analysis for the survival of *Pseudomonas fluorescens* (Sw5-RN) inoculated onto bare soil in both laboratory intact soil core microcosms and field plots. Populations are measured in colony forming units (CFU) remaining. Analysis of variance revealed the slopes, and the separation of the lines, are not significantly different at $p<0.05$. Each circle represents a single sample. ◑ Represents overlapping data points.

inoculation resulted in Pw-2R population counts between 4.27×10^3 and 1.10×10^4 $CFU \cdot g^{-1}$ fresh root for both laboratory microcosms and field plots. The first laboratory rhizosphere microcosm to have a population count below the limit of detection was sampled on day 122 after inoculation. On the next sampling day, (day 143), all of the samples from both the field plots and laboratory microcosms were below the limit of detection. Strain Pw-2R could not be isolated in either the field or laboratory microcosms 143 days after inoculation. A regular sampling schedule continued until day 186. No target organisms were identified during this period. Sampling stopped at this point until day 333.

Bacillus polymyxa Pw-2R is a rhizobacterium and it was expected that population counts would remain above the limit of detection for at least the first year. When the population counts for Pw-2R dropped below the level of detection, the theory of antibiotic masking was investigated. Sampling starting early the second season, on day 333, and all samples were plated with the original method of dispensing aliquots directly onto tryptic soy agar plates containing antibiotics along with cycloheximide and nystatin (TSA*). No Pw-2R target organisms were detected on this day and it was assumed that the populations were below the limit of detection.

On the following sampling day, day 354, the method for detecting antibiotic masking was employed. Each sample was originally plated onto tryptic soy agar plates containing cycloheximide and nystatin, and no antibiotics (TSA) as well as onto TSA* with antibiotics. The plates were allowed to incubate for 18 hours. *Bacillus polymyxa* Pw-2R colonies formed on the TSA plates only. The TSA plates were then

replicated onto plates containing antibiotics (TSA*) and allowed to incubate again for 24 to 36 hours. Pw-2R colonies grew on the TSA* plates and were enumerated at this point.

The use of replica plating to circumvent antibiotic masking continued until the final sampling day. The limit of detection varied with the amount of root weight sampled. A lower limit of detection was possible with larger root samples. The minimum level of detection was 2.1 $CFU \cdot g^{-1}$ of fresh root weight for the largest (43.37 g) field rhizosphere sampled. Population levels of Pw-2R above the limit of detection continued to be found in the field rhizospheres until day 417 and in the laboratory microcosm rhizospheres until the final day of sampling, day 489. The minimum level of detection for the laboratory rhizospheres was 2.3 $CFU \cdot g^{-1}$ of fresh root weight for the largest (39.52 g) lab rhizosphere sampled.

To compare the results of population decline between Pw-2R inoculated field rhizospheres and laboratory inoculated microcosm rhizospheres, regression analysis was used to compare slopes. The line of best fit for both field and microcosm data sets was a cubic polynomial regression (Figure 3). In general, the survival trend for Pw-2R populations inoculated into one year old hybrid spruce rhizospheres was a gradual decrease until population levels dropped below the limit of detection.

An analysis of variance was conducted using an F-ratio test to compare the lines of best fit for both field and microcosm Pw-2R populations. There was no significant difference ($p<0.01$) in the rate of population decline between Pw-2R field and laboratory inoculated rhizospheres (Appendix-Table A5). The length of survival of the Pw-2R inoculated in the rhizospheres of spruce seedlings in laboratory

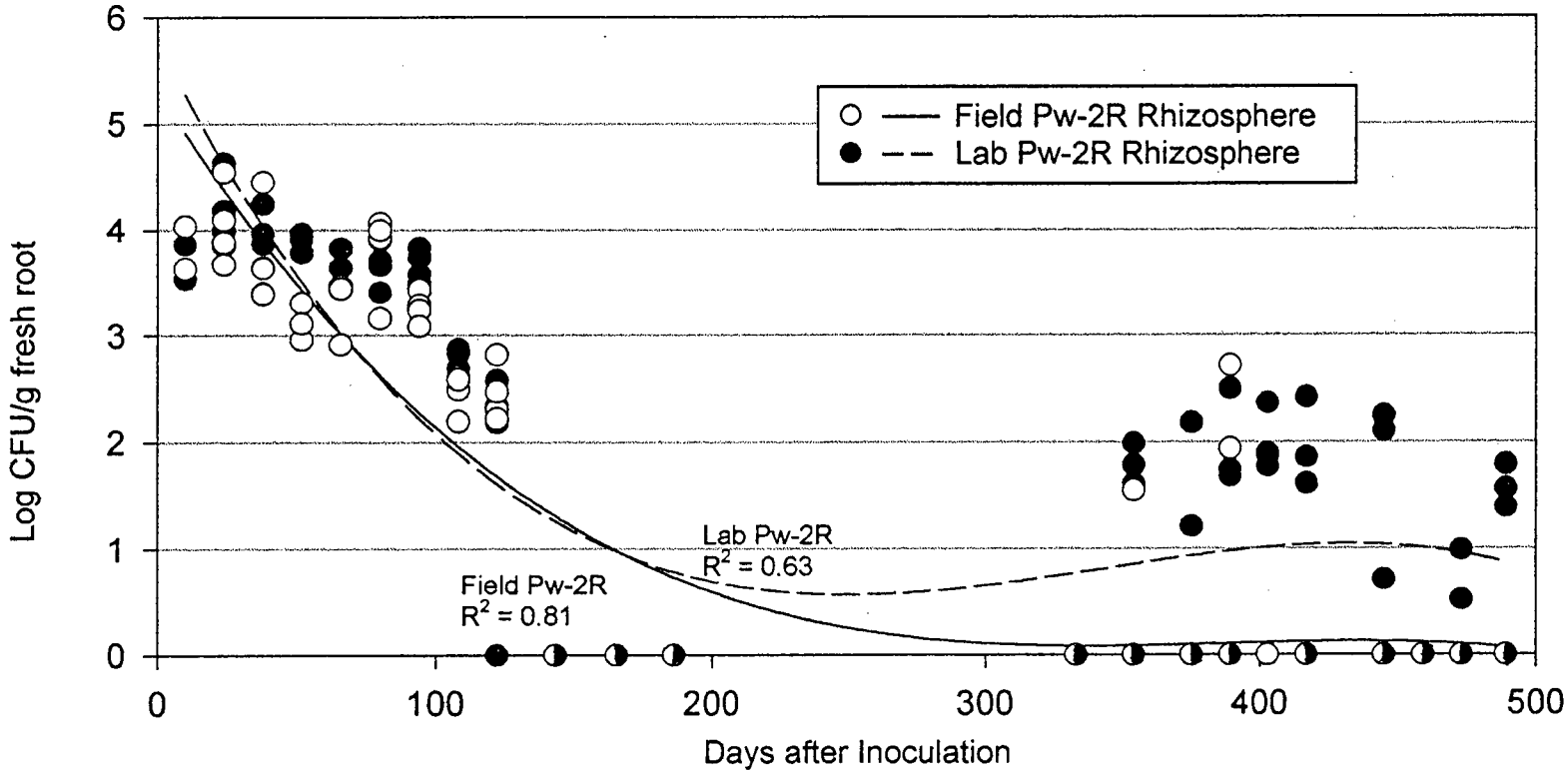

Figure 3. Regression analysis for the survival of *Bacillus polymyxa* (Pw-2R) inoculated into the rhizosphere of one year old hybrid spruce seedlings in both laboratory intact soil core microcosms and field plots. Populations are measured in colony forming units (CFU) remaining. Analysis of variance revealed the slopes are not significantly different at $p<0.01$. Analysis of variance further revealed that there is a significant separation ($p<0.01$) between the lines. Each circle represents a single sample. ◑ Represents overlapping data points.

microcosms was greater than the survival of the Pw-2R in the rhizospheres of spruce seedlings in the field plots. Testing for separation of regression slopes showed a significant difference ($p<0.01$) in the separation of the regression lines (Appendix-Table A6). Therefore the regression lines for the population decline of *Bacillus polymyxa* Pw-2R inoculated into field plot rhizospheres versus laboratory microcosm rhizospheres were parallel but were not coincidental.

The populations of P. *fluorescens* Sw5-RN in spruce seedling rhizospheres in field plots and laboratory microcosms followed a similar pattern to that of B. *polymyxa* Pw-2R (Figures 3 and 4). The inoculation density of Sw5-RN was approximately 1.0×10^7 colony forming units (CFU)$\cdot mL^{-1}$. Each seedling was inoculated by applying 5 mL of culture to the centre of the plug using a 10cc syringe and 18-gauge needle.

The populations of Sw5-RN continued to decline and were below the level of detection by day 143 in the field rhizospheres and by day 165 in the laboratory rhizospheres. The limit of detection varied with the amount of root weight sampled. A lower limit of detection was possible with larger root samples. The minimum level of detection was 1.4 CFU$\cdot g^{-1}$ fresh root weight for the largest (63.31 g) rhizosphere sampled.

Time of sampling for the Sw5-RN rhizospheres was identical to that of the Pw-2R rhizospheres. Sampling stopped on day 186 and there were no target organisms (Sw5-RN) identified on this day. All of the samples were originally plated on tryptic soy agar with100 mg$\cdot L^{-1}$ each of nalidixic acid and rifamycin along with the antifungal agents cycloheximide and nystatin (TSA**). Sampling resumed again on

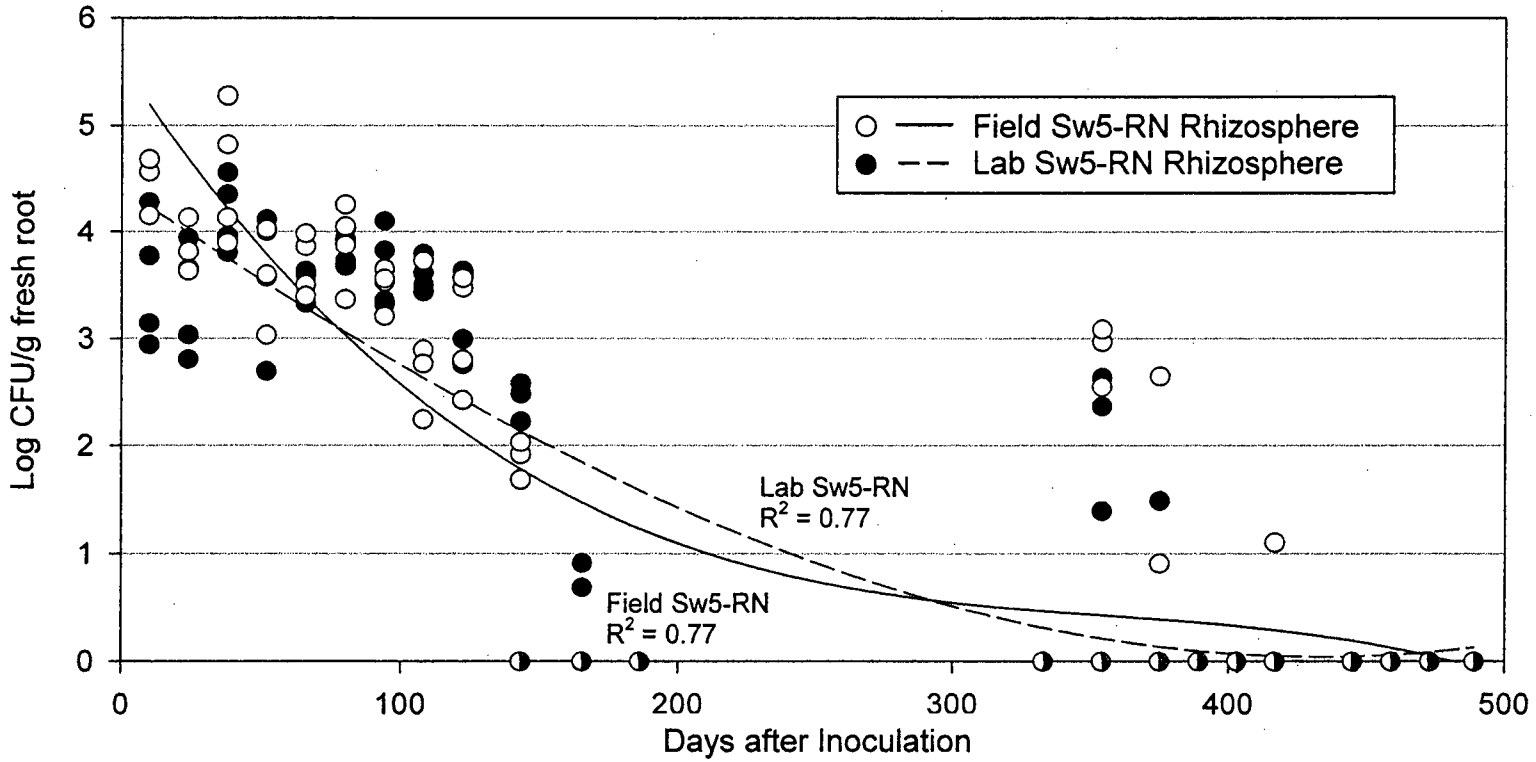

Figure 4. Regression analysis for the survival of *Pseudomonas fluorescens* (Sw5-RN) inoculated into the rhizosphere of one year old hybrid spruce seedlings in both laboratory intact soil core microcosms and field plots. Populations are measured in colony forming units (CFU) remaining. Analysis of variance revealed the slopes, and the separation of the lines are not significantly different at $p<0.05$. Each circle represents a single sample. ◑ Represents overlapping data points.

day 333 and continued until day 489.

Sampling for *Pseudomonas fluorescens* Sw5-RN on day 333 did not employ the use of the antibiotic plating method and no target colonies were isolated. On day 354, samples from the original dilutions were plated onto both TSA and TSA with100 $mg \cdot L^{-1}$ each of nalidixic acid and rifamycin along with the antifungal agents cycloheximide and nystatin (TSA**) to test for antibiotic masking. Target Sw5-RN colonies only grew on the TSA plates. When the colonies from the TSA plates were replicated onto TSA**, the same colonies were able to thrive. The same method of sampling continued until the final sampling day. Populations of Sw5-RN in the laboratory microcosm rhizospheres dropped below the limit of detection (3.2 $CFU \cdot g^{-1}$ fresh root weight) after sampling on day 375 and were never again recovered. Populations of Sw5-RN in the field plot rhizospheres dropped below the limit of detection (1.4 $CFU \cdot g^{-1}$ fresh root weight) after sampling on day 417 and were never again recovered.

The line of best fit for the population decline for both the field and laboratory microcosms followed a cubic polynomial regression (Figure 4). Statistical testing by analysis of variance using the F-ratio test showed no significant difference ($p<0.05$) in the slopes of the two Sw5-RN regression lines (Appendix-Table A7). Therefore, there was no significant difference between the rates of population decline of *Pseudomonas fluorescens* Sw5-RN inoculated into field and laboratory rhizospheres. Further analysis revealed no significant difference ($p<0.05$) in the separation of the cubic regression lines (Appendix-Table A8). Therefore the regression lines for the population decline of *Pseudomonas fluorescens* Sw5-RN

inoculated into field plot rhizospheres versus laboratory microcosm rhizospheres were not only parallel but also coincidental.

3.3 Internal Plant Colonization Assay:

When internal root and shoot colonization was evaluated using the surface sterilization-dilution plating assay there were no target organisms identified. No *Bacillus polymyxa* Pw-2R were detected inside the roots or shoots of the spruce seedlings. Prior to the use of the antibiotic resistance plating method, the initial 50% strength tryptic soy agar (TSA) plates amended with cycloheximide (100 $mg \cdot L^{-1}$) and nystatin (50 $mg \cdot L^{-1}$) displayed growth of what looked morphologically to be Pw-2R. After replica plating onto 50% strength TSA amended with 200 $mg \cdot L^{-1}$ rifamycin, 100 $mg \cdot L^{-1}$ cycloheximide and 50 $mg \cdot L^{-1}$ nystatin, no *Bacillus polymyxa* Pw-2R colonies grew.

3.4 Plant Growth Promotion Assay:

The result of inoculation with either *Bacillus polymyxa* Pw-2R or *Pseudomonas fluorescens* Sw5-RN on seedling growth was minimal.

The field inoculation data was plotted using linear regression (Figure 5) and analyzed using analysis of variance (ANOVA) with an F-ratio test. The results indicated there was no significant difference ($p<0.05$) in the rate of total biomass accumulation (Appendix-Tables A9, A11, and A13), shoot biomass accumulation, or root biomass accumulation between seedlings inoculated with Pw-2R, Sw5-RN, or left uninoculated. Further ANOVA using an F-ratio test revealed the linear regression

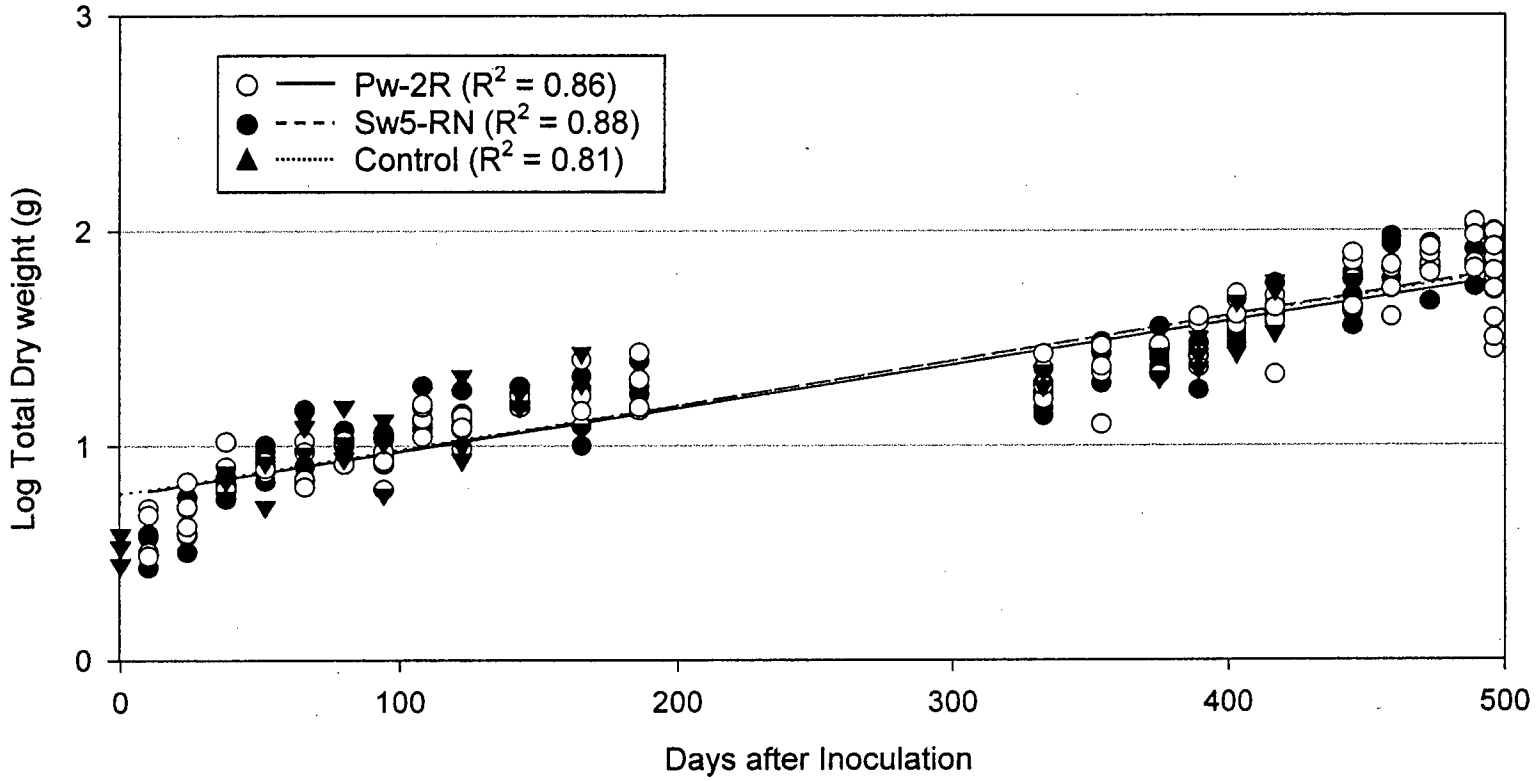

Figure 5. Regression analysis for the total biomass accumulation of FIELD planted one year old hybrid spruce seedlings inoculated with *Bacillus polymyxa* (Pw-2R) and *Pseudomonas fluorescens* (Sw5-RN) into the rhizosphere. Analysis of variance compared the regression slopes and the separation of the lines against control seedlings (non-inoculated). Analysis of variance revealed the slopes, and the separation of the lines are not significantly different at $p<0.05$. Each point represents a single sample.

lines are not significantly separated ($p<0.05$) and are coincidental for the rate of total biomass accumulation (Appendix-Tables A10, A12, and A14), shoot biomass accumulation and root biomass accumulation. Analysis of variance using Fisher's LSD revealed the only difference in sampling day treatment means occurred on the final day of sampling and showed the total dry biomass and the shoot dry biomass of the control to be significantly greater ($p<0.05$) than either inoculated treatment (Figure 6). There was no other sampling day that revealed any significant difference.

The ANOVA using an F-ratio test for the laboratory results indicated no significant difference ($p<0.05$) in the rate of total biomass accumulation (Appendix-Tables A15, A17, A19), shoot biomass accumulation, or root biomass accumulation between seedlings inoculated with Pw-2R, Sw5-RN, or left uninoculated. The analysis also indicated no significant difference ($p<0.01$) in the separation between the linear regression lines. The linear regressions are coincidental for the rate of total biomass accumulation (Appendix-Tables A16, A18, A20), shoot biomass accumulation and root biomass accumulation.

Analysis of variance of the treatment means using Fisher's LSD on the final day of sampling indicated Pw-2R inoculated seedlings had significantly greater ($p<0.05$) total biomass than either Sw5-RN inoculated seedlings or the control seedlings (Figure 7). Further analysis indicated the Pw-2R inoculated seedlings had significantly greater ($p<0.05$) shoot and root biomass than either the Sw5-RN inoculated seedlings or the control on the final day of sampling.

Analysis of variance on day 445 (Figure 8) indicated that the Pw-2R and Sw5-RN inoculated seedlings had a significantly greater ($p<0.05$) increase in total

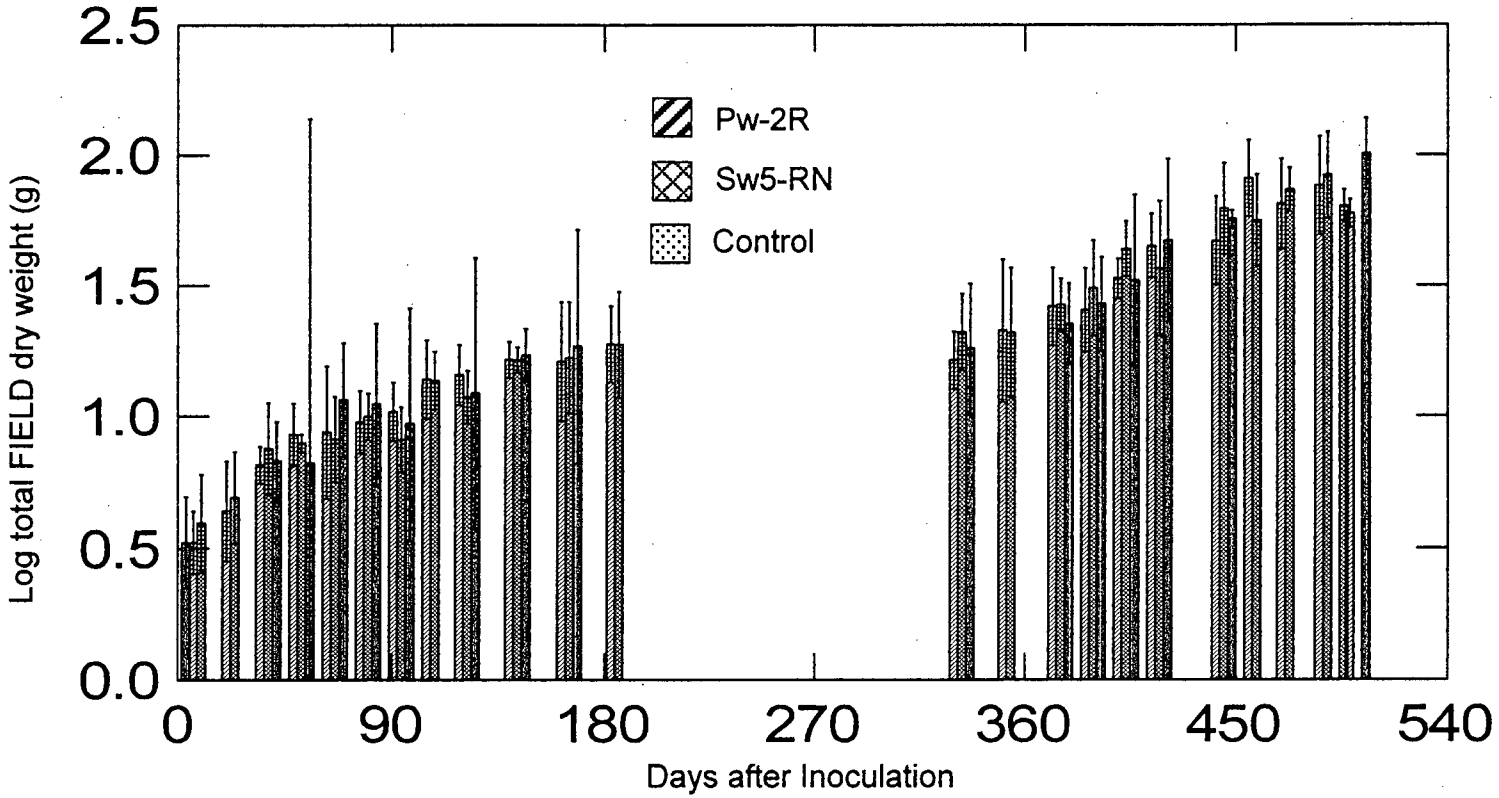

Figure 6. Mean biomass accumulation for *Bacillus polymyxa* Pw-2R and *Pseudomonas fluorescens* Sw5-RN inoculated one-year-old hybrid spruce seedlings. Control seedlings were left uninoculated. Seedlings were planted in field plots. Error bars show standard error of the mean (95%). The only sampling day to show a significant difference in biomass accumulation was the final day in which the control seedlings had significantly greater biomass ($p<0.05$).

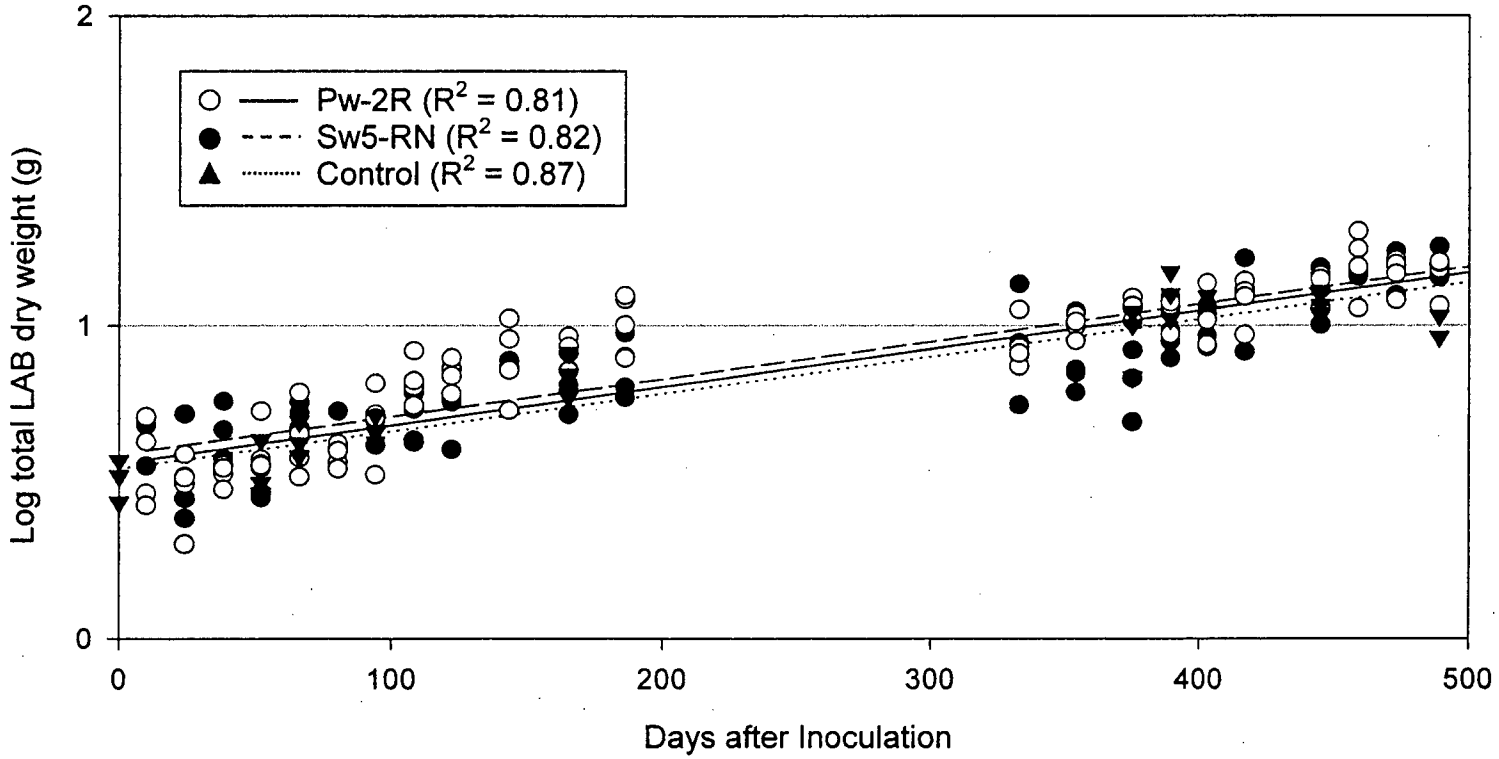

Figure 7. Regression analysis for the total biomass accumulation of laboratory MICROCOSM planted one year old hybrid spruce seedlings inoculated with *Bacillus polymyxa* (Pw-2R) and *Pseudomonas fluorescens* (Sw5-RN) into the rhizosphere. Analysis of variance compared the regression slopes and the separation of the lines against control seedlings (non-inoculated). Analysis of variance revealed the slopes, and the separation of the lines are not significantly different at $p<0.05$. Each point represents a single sample.

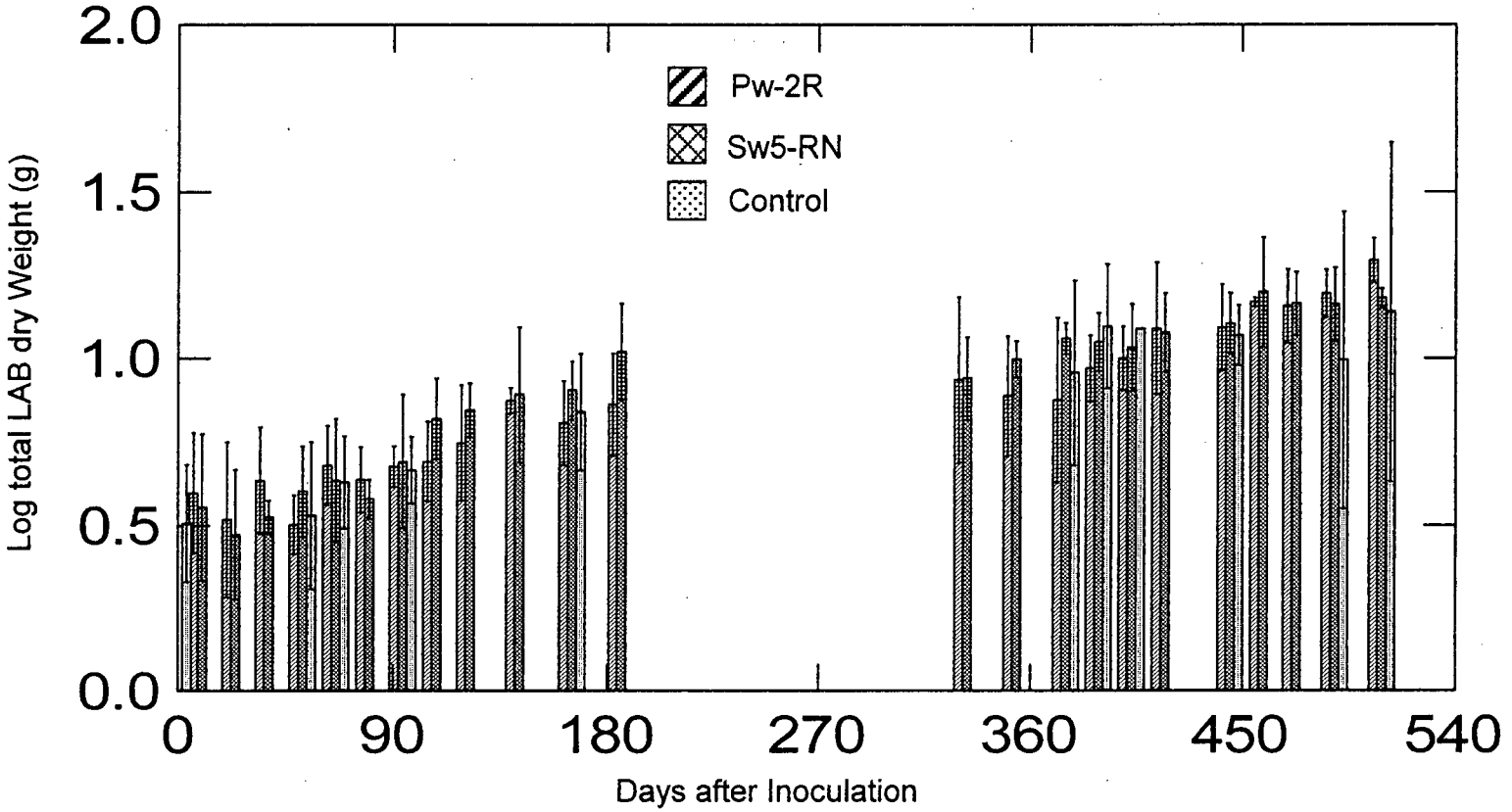

Figure 8. Mean biomass accumulation for *Bacillus polymyxa* Pw-2R and *Pseudomonas fluorescens* Sw5-RN inoculated one year old hybrid spruce seedlings. Control seedlings were left uninoculated. Seedlings were planted in laboratory microcosms. Error bars show standard error of the mean (95%). Significant differences were found on only two sampling days. Analysis of variance of the treatment means on the final day of sampling indicated Pw-2R inoculated seedlings had significantly greater ($p<0.05$) total biomass than either Sw5-RN inoculated seedlings or the control seedlings. On day 445 the Pw-2R and Sw5-RN inoculated seedlings had a significantly greater ($p<0.05$) total biomass than the control.

biomass than the control. The same results occurred for the increase in shoot and root biomass with the Pw-2R and Sw5-RN inoculated seedlings having a significantly greater ($p<0.05$) increase. These were the only two sampling days to reveal any significant differences in biomass accumulation.

Section 4.
Discussion:

Social implications and public confidence in the rapid advancement of molecular biology are crucial areas of concern. Recombinant DNA techniques have been developed to improve crop production and to enhance degradation of environmental pollutants (Halvorson et al. 1985). Evidence suggests that the public perception of the risks associated with genetically engineered microorganisms (GEM's) is very volatile (Stewart-Tull et al. 1992). Public attitudes towards risk suggest the need for standardization of microcosm design for risk assessment of new products that result from genetic engineering.

Microcosms are intended to provide a standardized model ecosystem to simulate the extremely complex interaction of physical, chemical and biological components of terrestrial environments. Prerelease microcosm studies may be used to determine if genetic modifications introduced into a microorganism are likely to create any unacceptable risks before that organism is released into the environment (Krimsky et al. 1995). Soil microcosms can be an invaluable tool for the study of microorganisms in natural soil conditions. However, in order to become a standard assessment tool, a microcosm must be proven to accurately simulate field conditions.

4.1 Microcosm Validation:

4.1.1 Bare Soil:

One of the objectives of this research was to determine how accurately results obtained in microcosms reflected those obtained in the field. This was

accomplished by assessing the fate of *Bacillus polymyxa* strain Pw-2R as well as *Pseudomonas fluorescens* strain Sw5-RN, in bare soil and in the rhizosphere of spruce seedlings, in both field plots and in intact soil core microcosms. Pw-2R as well as Sw5-RN have been shown to induce growth stimulation in conifer species and have been classified as plant growth promoting rhizobacteria (PGPR) (Chanway and Holl 1993a, Chanway and Holl 1994).

The initial inoculation density for Pw-2R was approximately 2.0×10^6 colony forming units (CFU)$\cdot g^{-1}$ fresh soil. The population of Pw-2R continued to decline for the first two sampling days. This was followed by an increase in capture size on sampling day 24 in both field plots and laboratory microcosms and then a rapid decline until population levels dropped below the limit of detection (Figure 1). Analysis of variance using Fisher's LSD revealed that the increase in capture size of Pw-2R on day 24 was not significant.

A similar trial was performed using *Pseudomonas fluorescens* Sw5-RN inoculated onto bare soil in field plots and laboratory microcosms and similar results from the Pw-2R trial were obtained. The population of Sw5-RN continued to decline immediately after inoculation until population levels were below the limit of detection (Figure 2). The initial recovery rate of Sw5-RN on the first sampling day was slightly lower than the expected level. This initial recovery rate was not significantly lower, ($p<0.05$), than the expected recovery level.

To compare the population decline of Pw-2R inoculated onto bare soil field plots with bare soil microcosms, regression analysis was used to compare the population decline over time (Kozak 1964). From both a biological and statistical

perspective, the line of best fit for both field and microcosm data sets was a cubic polynomial regression. In general, the survival trend for Pw-2R inoculated onto bare soil was a slight increase followed by a steep decrease until population levels dropped below the limit of detection.

Using an F-ratio test, the analysis of variance (ANOVA) allowed an accurate comparison between the slope and position of the regression lines for Pw-2R inoculated field plots and laboratory microcosms. The results indicate that the bare soil laboratory microcosms can be used to accurately predict the survival of *Bacillus polymyxa* Pw-2R inoculated onto bare soil in the field at this location.

Regression analysis was further used to compare the results of population decline between Sw5-RN inoculated bare soil field plots and laboratory inoculated bare soil microcosms. The line of best fit for both field and microcosm data sets was a cubic polynomial regression. Using an F-ratio test, the analysis of variance (ANOVA) revealed no significant difference between the slopes or the separation of the two regression lines. These results indicate that the bare soil microcosms can be used to accurately predict the survival of *Pseudomonas fluorescens* Sw5-RN inoculated onto bare soil in the field at this location.

4.1.2 Possible Reasons for Bacterial Population Decline:

Bacillus polymyxa Pw-2R was originally isolated from internal root tissue of a naturally regenerating pine seedling (Shishido et al. 1995), and is a plant growth promoting endophyte that is able to live inside plant tissue. It is known that the number of soil organisms at successive distances from a root surface is inversely correlated with increased distance (Paul and Clark 1989). The region under direct

influence from plant roots, the rhizosphere, contains large amounts of easily oxidizable nutrients that support bacterial growth. Without the availability of nutrients it would be expected that population levels would decrease. Pw-2R is likely adapted to colonize plant tissue, therefore with the lack of a suitable host, the rapid decrease in population of Pw-2R inoculated onto bare soil was not surprising.

Prior to taking the intact soil core microcosms from the field, and prior to inoculation, a cultivator was used to plough under any remaining plant material in the field. Being placed under the black polyvinyl sheeting had previously killed most of the plant material. The cultivating would have chopped up and randomly distributed any remaining plant material, including root systems. The brief increase in *Bacillus polymyxa* Pw-2R population may have been caused in part by the Pw-2R becoming established in microsites that contained easily oxidizable nutrients. These nutrients may have become accessible as a result of chopping up the root systems of the original plant matter in the field. The possibility exists that once this nutrient store had been depleted the population levels of Pw-2R rapidly decreased. The field plots and laboratory microcosms were all weeded by hand to keep them free of any plant growth through the duration of the experiment; therefore new root systems were kept to a minimum. Without the root systems, the level of available nutrients for these rhizobacterial populations would be extremely low.

The possibility also exists that competition from other microorganisms caused the overall rapid decline in Pw-2R population numbers. A recent study has shown the partial inhibition of growth-promoting effects of Pw-2R due to the presence of other rhizobacteria (Bent and Chanway 1998). Recent studies have shown PGPR

efficacy on interior spruce seedling growth was greatest when the seedlings were grown in forest soil and inoculated with rhizosphere bacteria that originated from the same site as the spruce seed (O'Neill et al. 1992a). Further studies with PGPR have illustrated significant soil community responses to PGPR inoculation (Shishido and Chanway 1998). Shishido (1998) demonstrated that soil community responses depended on the origin of the soil and the presence of seedlings.

The indigenous soil community present at the University of British Columbia may have caused a negative impact on the Pw-2R population levels. The rapid decline was seen in both inoculated field and laboratory microcosms, therefore the soil conditions were likely the same in both.

As is true with the *Bacillus polymyxa* Pw-2R, *Pseudomonas fluorescens* Sw5-RN is likely adapted to colonize plant tissue, therefore with the lack of a suitable host the rapid decrease in population of Sw5-RN inoculated onto bare soil was not surprising. The survival trend of the Sw5-RN populations in the field and microcosms did not show any increase after inoculation. Therefore, any nutrients available may not have been as readily accessible as they were for the Pw-2R, or may not have been the nutrients necessary for the survival of the Sw5-RN. Competition from other microorganisms may also have been a cause for the overall rapid decline in Sw5-RN population numbers.

4.1.3 Antibiotic Masking:

Previous studies have been completed using the same microcosm with the genetically engineered microorganism (GEM) *Pseudomonas chloroaphis* 3732RN-L11 and Canadian hard red spring wheat; *Triticum aestivum*; cultivar A.C. Karma

(Angle et al. 1995). One of the goals of this research was to validate the microcosm for use with other organisms and other plants.

As a general trend, population levels of Pw-2R declined following inoculation until the population size fell below the limit of detection (Figure 3). The first rhizosphere sampling day occurred on May 13,1998 and continued every third week until the final rhizosphere sampling day for that year, November 4 (day 186). Population levels declined from the initial sampling day until levels were below the limit of detection on day 122 in both field plot rhizospheres and laboratory microcosm rhizospheres. During the sampling period, between day 122 and day 186, no target organisms (Pw-2R) were detected and all samples were assumed to be below the limit of detection.

A standard method to recover specific bacterial strains after environmental release is the use of spontaneously generated antibiotic resistant strains. It is an inexpensive and effective method for marking bacteria for easy recovery using cultural methods (Kloepper and Beauchamp 1992). Bacteria become resistant to antibiotics in different ways. Resistance mechanisms include bypassing the metabolic step affected by the antibiotic, overproducing an enzyme or product to detoxify the antibiotic, altering the structure of a target enzyme, and developing an uptake system for destruction or modification of the antibiotic (Dawes and Sutherland 1991). It is assumed in these methods that the antibiotic resistant microorganism retains its ability to thrive in the presence of specific levels of the antibiotic.

Recent studies on internal plant colonization by endophytic bacteria marked with antibiotic resistance have discovered what seems to be the temporary loss of the ability to thrive in the presence of specific levels of antibiotics. This loss has been termed antibiotic masking (McInroy et al. 1996). McInroy (1996) treated plants with rifampicin-resistant endophytic bacteria (rif+ mutants), and attempted reisolation of these bacteria after 3-14 days on agar amended with rifampicin (RTSA) with no success. However, colonies initially isolated from the same sample on agar with no antibiotics and then transferred to RTSA grew within 18 hours. McInroy (1996) does not attempt to explain the causes for the observed antibiotic masking other than the possibility that internal plant extracts may be affecting the ability of the endophyte to grow on RTSA. McInroy (1996) does go on to state that antibiotic masking was not encountered when isolating bacteria from external root surfaces.

From day 10 until day 186 the diluted samples of *Bacillus polymyxa* Pw-2R were all plated on tryptic soy agar containing 200 $mg \cdot L^{-1}$ rifamycin, 100 $mg \cdot L^{-1}$ cycloheximide and 50 $mg \cdot L^{-1}$ of nystatin (TSA*). Pw-2R population counts from sampling days 122 to 186, apparently dropped below the limit of detection. However, with the use of sample plating to uncover antibiotic resistance, viable populations were detected on sampling days 354 and 389 in the field rhizospheres and days 354 through to the final sampling day (489) for laboratory rhizospheres.

Bacillus polymyxa Pw-2R may have been present during sampling days when population counts of zero occurred in the field rhizospheres. The organisms may have just been undetectable, as they could have been below the limit of detection but above zero. Other studies have shown that large bacterial (PGPR) population

levels may not be needed to result in a plant growth response (Holl and Chanway 1992). On the other hand, the populations may have just completely died out in the second sampling season in most rhizospheres.

Similar results were obtained when *Pseudomonas fluorescens* Sw5-RN was inoculated into the root plugs of the spruce seedlings in the laboratory microcosms and in the field.

Sampling of *Pseudomonas fluorescens* Sw5-RN began on day 10 and continued until day 489. The first rhizosphere sampling day was May 13,1998 and the final rhizosphere sampling day for that year was November 4 (day 186). Population levels of Sw5-RN increased slightly following the inoculation until day 38, then continued to decline until population levels were below the limit of detection (Figure 4). *Pseudomonas fluorescens* Sw5-RN was originally isolated from the rhizosphere of hybrid spruce (*Picea glauca x engelmannii*) seedlings (Shishido et al. 1996b). The seedlings used in this experiment were hybrid spruce and the microenvironment that the inoculum was first presented with may have caused the initial population levels to increase slightly. An analysis of variance concluded that the initial rise in population was not significant in either the laboratory microcosm rhizospheres or the field plot rhizospheres. The slight increase in Sw5-RN population may be explained by the fact that *Pseudomonas* species are much more vigorous root colonizers than are *Bacillus* species (Kloepper et al. 1989). Kloepper (1989) explains that the rhizosphere is preferentially inhabited by pseudomonads, therefore *Pseudomonas fluorescens* Sw5-RN inoculated into the rhizosphere of the spruce seedlings may have had an advantage in survival.

As with the Pw-2R, the Sw5-RN inoculum was injected into the middle of the root plugs. Microsites where exudates and root derived organic material will be available are favourable sites in which microbial growth and competition will occur (Metting 1993). Thus the lack of nutrients or competition from indigenous microorganisms in the rhizosphere may have caused the decline in both the Pw-2R and Sw5-RN populations.

The first time the Sw5-RN population levels in the field and laboratory rhizospheres dropped below the limit of detection was on day 143. At the beginning of sampling in the second season (day 333), testing for antibiotic masking occurred.

The population counts between sampling days 143 to 186 that were assumed to be below the limit of detection might have just been displaying antibiotic masking. With the use of antibiotic resistance plating methods, viable populations were detected on sampling days 354 and 417 in the field rhizospheres and days 354 and 375 in laboratory rhizospheres. Populations in the field and laboratory rhizospheres may have been present after these sampling days but undetectable as they may have been below the limit of detection but above zero, or they may have dropped to zero in the second sampling season.

The *Bacillus polymyxa* Pw-2R cells were originally cultured for inoculation in broth amended with 200 $mg \cdot L^{-1}$ rifamycin. The mechanism(s) that allowed the cells to thrive in this level of antibiotic may have been suppressed after the Pw-2R was inoculated into the rhizosphere of the spruce seedlings. Plating of all samples from the original dilutions onto TSA and TSA amended with antibiotics, (TSA*), showed that the only Pw-2R populations able to grow initially were the colonies plated onto

the TSA containing no antibiotics. When the TSA plates were replicated onto the TSA* plates containing the antibiotics, Pw-2R colonies were able to grow.

The *Pseudomonas fluorescens* Sw5-RN cells were originally cultured for inoculation in tryptic soy broth amended and 100 $mg \cdot L^{-1}$ each of nalidixic acid and rifamycin. The mechanism(s) that allowed the cells to thrive in this level of antibiotic may have been suppressed similarly to the Pw-2R mechanism(s).

One explanation for the antibiotic masking may be that there was a decrease in the necessity of the Pw-2R and Sw5-RN to overproduce inhibitory enzymes or products, as there were no antibiotics present in the rhizosphere of the seedlings. Rifamycin acts by inhibiting bacterial RNA synthesis by binding strongly to the beta subunit of DNA-dependant RNA polymerase, preventing the attachment of the enzyme to DNA and thus blocking initiation of RNA transcription (McEvoy 1995). Nalidixic acid appears to act by inhibiting bacterial DNA synthesis, probably by interfering with DNA polymerization by inhibiting DNA gyrase (McEvoy 1995).

Bacillus polymyxa Pw-2R may produce compounds that bind or break down the rifamycin before it is able to block RNA transcription. The resistance of Pw-2R to rifamycin may not have been lost but only suppressed. *Pseudomonas fluorescens* Sw5-RN may similarly produce compounds enabling resistance to both rifamycin and nalidixic acid.

The colonies of both Pw-2R and Sw5-RN that were replicated onto TSA plates containing antibiotics would contain a logarithmic population of cells. This may allow the colonies to grow on the TSA plates containing antibiotics, as some cells would be initially physically shielded from the high levels of antibiotics in the plates.

Colony formation did take longer after the colonies had been replicated onto TSA plates containing antibiotics. This lag in time may have indicated a slow response of the cells due to the levels of antibiotics. Being subjected to the lower levels of antibiotics by being physically shielded in the colonies may have allowed some organisms time to produce the necessary compounds required for antibiotic resistance. This is only speculation and further studies need to be undertaken in order to determine the reasons for the antibiotic masking.

The fact that these two different antibiotic resistant organisms, Pw-2R and Sw5-RN, displayed antibiotic masking properties shows that care should be taken when attempting to recover antibiotic resistant organisms after release. Further studies should be undertaken to better understand the reasons the antibiotic masking.

4.1.4 Rhizosphere:

Using an F-ratio test, the analysis of variance (ANOVA) allowed an accurate comparison between the slope and position of the regression lines for the population decline of Pw-2R inoculated field plot rhizospheres and laboratory microcosm rhizospheres. There was no significant difference ($p<0.01$) between the slopes of the regression lines. The statistical analysis indicated that the lines were not coincidental. The length of survival of the Pw-2R inoculated into the rhizospheres of laboratory microcosms was greater than the survival of the Pw-2R inoculated into the field rhizospheres.

The results indicate that the laboratory microcosms containing Pw-2R inoculated spruce seedlings can be used to accurately predict the rate of population

decline of the Pw-2R inoculated into the rhizosphere of the spruce seedlings in the field at this location. The results also indicate that the microcosms can not be used to predict the final day when the *Bacillus polymyxa* Pw-2R population reaches zero in the field rhizospheres. Therefore the microcosms have the ability to conservatively predict the survival rate of the *Bacillus polymyxa* Pw-2R inoculated into the field rhizospheres.

Similarly to Pw-2R, in order to analyze the results of population decline between Sw5-RN inoculated field rhizospheres and laboratory inoculated microcosm rhizospheres, regression analysis was used to compare slopes.

The analysis of variance (ANOVA) using an F-ratio test allowed an accurate comparison between the slope and position of the regression lines for Sw5-RN inoculated field plot rhizospheres and laboratory microcosm rhizospheres. The rate of population decline between the laboratory microcosm rhizospheres and the field plot rhizospheres was not significantly different. The ANOVA further revealed that the regression lines are coincidental.

The results indicate that the laboratory microcosms containing *Pseudomonas fluorescens* Sw5-RN inoculated into the rhizosphere of the spruce seedlings can be used to accurately predict the survival of the Sw5-RN inoculated into the rhizosphere of the spruce seedlings in the field at this location. The results further revealed that the laboratory microcosms could be used to predict the final day when the *Pseudomonas fluorescens* Sw5-RN population reaches zero in the field rhizospheres.

Therefore, the trials with *Bacillus polymyxa* Pw-2R and *Pseudomonas fluorescens* Sw5-RN inoculated onto this bare soil and into the rhizosphere of these spruce seedlings validate the use of this intact soil core microcosm to predict the survival of microorganisms that may be released to the environment.

4.2 Internal Plant Colonization:

Bacillus polymyxa Pw-2R is a plant growth promoting rhizobacteria that has been detected inside roots of hybrid spruce seedlings using a surface sterilization-dilution plating assay (Shishido et al. 1999). Shishido et al (1999) demonstrated the ability of Pw-2R to enter spruce root tissues, establishing endophytic populations after seed inoculation without causing visible symptoms of disease. The results presented here indicate that the Pw-2R did not colonize the internal root or shoot tissues of the spruce seedlings. Soil conditions and indigenous soil organisms may have reduced or eliminated the ability of the Pw-2R populations to enter the root and travel to the shoot tissues. Rhizobacteria present at this site may have inhibited the ability of the Pw-2R to enter the roots, in turn inhibiting the growth promoting effects (Bent and Chanway 1998). This may have also been caused by predation, or other negative interactions occurring before the Pw-2R had a chance to establish sustainable internal populations (Shishido et al. 1999). Chanway (1998) suggested that a period of growth in a controlled environment to establish endophytic populations may have great potential for reforestation. The results presented here indicate that the Pw-2R were not able to establish an endophytic population when inoculated after being planted. The conception of pre-inoculating seedlings with

growth promoting rhizobacteria in a controlled environment, such as a nursery, before use in reforestation, should be investigated further.

4.3 PGPR Efficacy:

Bacillus polymyxa Pw-2R and *Pseudomonas fluorescens* Sw5-RN are both plant growth promoting rhizobacteria (PGPR) (Chanway and Holl 1993a, Chanway and Holl 1994). The results from the current trial indicate that PGPR strains *Bacillus polymyxa* Pw-2R and *Pseudomonas fluorescens* Sw5-RN inoculated into the rhizosphere of spruce seedlings did not increase the biomass accumulation rate when the seedlings were in a relatively stress free environment. The only difference in the field plots occurred on the final sampling day; the mean for the dry biomass of the control seedlings was significantly greater than either of the inoculated seedling treatments.

Results for the rate of seedling biomass accumulation for Pw-2R or Sw5-RN inoculated seedlings in the laboratory microcosms also displayed no significant difference. When individual sampling days were examined, only two days showed differences in treatment means. Each showed a significantly greater shoot and root biomass accumulation in the Pw-2R inoculated seedlings.

Several theories have been put forth to attempt to explain the mechanisms for PGPR activity. Chanway (1997) has reviewed these mechanisms and divides them into direct and indirect. The review suggests that direct mechanisms occur when bacteria produce a metabolite or compound that stimulates plants to grow independently of other soil organisms. Lack of direct mechanisms may be the

reason for the lack of growth promotion in the seedlings inoculated with either Pw-2R or Sw5-RN. In order to induce growth promotion, seedlings may need one or a set of precursors produced by the PGPR in order to create a growth-promoting hormone. Recent studies have shown specific plant hormones (auxins) can be produced by the microbial transformation of an available precursor (Arshad and Frankenberger 1991). If Pw-2R or Sw5-RN promote growth in seedlings by synthesizing growth hormones, the precursor molecule(s) that may be necessary may not have been present in the soil used for these trials. Another suggestion would be that plant growth promotion may require a balance of different growth hormones and the entire set of hormones necessary may not have been present in the soil used for these trials.

Chanway's (1997) review suggests plant growth promotion can also be caused by indirect methods involving bacteria which affect other factors that in turn stimulate plant growth. Indirect methods include increased mycorrhizal infection and suppression of deleterious bacteria. However, mycorrhizal interactions or the lack thereof were not considered in these trials.

Recent work suggests PGPR strains may inhibit the activity or growth of indigenous, plant growth inhibiting rhizosphere microorganisms (Kloepper 1993). The presence of the PGPR would therefore decrease the negative impact of the deleterious organisms and therefore allow increased growth of the seedlings. There may have been no deleterious organisms present in the soils used for these trials; therefore there may have been no negative effects to overcome.

A similar answer for the lack of growth promotion may also involve the indigenous bacterial populations. If the Pw-2R or the Sw5-RN were producing compounds such as growth hormones, the indigenous populations may have been depleting those compounds as soon as they were being created. Recent studies with microcosms have shown soil bacterial populations can be significantly altered and large shifts in carbon substrate utilization profiles can be caused in response to PGPR inoculation (Shishido and Chanway 1998).

In addition, plant growth promoting rhizobacteria may only cause significant increases in biomass accumulation when the seedlings are stressed. Recent studies have illustrated a greater PGPR effect shown in interior spruce and lodgepole pine seedlings that were grown at sites of lower productivity (Chanway and Holl 1993b, Chanway and Holl 1994). Chanway hypothesized that the PGPR inoculum may be more beneficial at sites that have harsh growing conditions.

Further investigation into the relationships of Pw-2R and Sw5-RN with the indigenous soil community in the field is necessary to understand the effects of these plant growth promoting rhizobacteria. Many complicated interactions are involved in this system of microbial plant growth promotion. Further work must be undertaken in order to answer these questions.

5. CONCLUSIONS:

The data generated in the experiments presented here confirm the hypothesis that the intact soil core microcosm can be used to accurately predict the fate of *Bacillus polymyxa* Pw-2R and *Pseudomonas fluorescens* Sw5-RN inoculated onto a bare field soil. The data confirmed the intact soil core can be used to predict the rate of population decline in the field as well as the day at which the population drops to zero in field inoculated soil.

The data also confirm that the *Bacillus polymyxa* Pw-2R and *Pseudomonas fluorescens* Sw5-RN populations declined more gradually in the rhizosphere of the seedlings. The intact soil core microcosms were able to predict the rate of population decline of *Bacillus polymyxa* Pw-2R and *Pseudomonas fluorescens* Sw5-RN inoculated into field rhizospheres. The microcosms were able to predict the day at which the population of *Pseudomonas fluorescens* Sw5-RN reached zero in the field plots but were not successful in predicting the same for the *Bacillus polymyxa* Pw-2R. However, the microcosms were able to conservatively predict the final day in which the field populations of both *Bacillus polymyxa* Pw-2R and *Pseudomonas fluorescens* Sw5-RN reached zero.

The testing of the predictive ability of the intact soil core microcosm revealed that this small, inexpensive and simple tool may be appropriate for risk assessment of microorganisms that might eventually be introduced to a soil or rhizosphere environment. Validation with additional soils, microorganisms and plants should be undertaken for this microcosm to be accepted as a standard for risk assessment but these results show great promise.

Two spontaneously generated antibiotic resistant strains of plant growth promoting rhizobacteria (PGPR) were used in these trials. The technique of using antibiotic resistant strains has been commonly used as it allows easy recovery of inexpensively marked bacteria using cultural methods. Recent studies have discovered what seems to be the temporary loss of the ability of antibiotic resistant strains to thrive in the presence of those antibiotics. This same loss was discovered in the two PGPR strains used in these trials. These results further confirm previous observations that antibiotic resistant bacteria that fail to grow upon primary isolation on agar media with antibiotics will grow on those same plates if first isolated on the same agar media without antibiotics. Previous studies have only observed this when isolating bacteria from within roots or stems. Antibiotic masking has been observed here for the first time when isolating antibiotic resistant bacteria from external root surfaces. Future work using antibiotic resistance as a method of selective isolation for soil microorganisms should be designed to include methods for identifying the possibility of antibiotic masking.

Since *Bacillus polymyxa* Pw-2R is a plant growth promoting endophyte it was hypothesized that colonies would be isolated from internal shoot and root tissues. Using the surface sterilization-dilution plating assay there were no Pw-2R organisms identified in any of the internal shoot or root tissues. The results of these trials indicate that the Pw-2R were not able to establish an endophytic population when inoculated after the seedlings had been planted. Previous research suggests that endophytic PGPR may be able to establish internal populations more readily before being subjected to competition, predation, or other negative interactions (Shishido et

al. 1999). Therefore, the *Bacillus polymyxa* Pw-2R may have had a better opportunity to establish an endophytic population if it had been inoculated into the rhizosphere prior to the seedlings being planted.

Finally, it was hypothesized that seedlings in the field or in the intact soil core microcosms inoculated with either *Bacillus polymyxa* Pw-2R or *Pseudomonas fluorescens* Sw5-RN would show an increase in biomass. Both Pw-2R and Sw5-RN have previously been shown to promote the growth of conifer seedlings and studies indicated that the positive effects of a single inoculation at planting can extend at least through the second year of growth (Chanway et al. 1997). The results from these trials indicated spruce seedling growth was not significantly enhanced by inoculation with Pw-2R or Sw5-RN in the field rhizospheres. Many complex interactions are undoubtedly involved in the mechanisms of plant growth promotion by rhizobacteria. Positive results seem to be linked with harsh growing sites and interactions with indigenous microorganisms in the primary soil from which the PGPR were originally isolated. Further investigation is necessary to understand the complicated interactions involved in the processes of plant growth promoting rhizobacteria.

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APPENDIX 1

Statistical Analysis

Table A1. Analysis of variance and F-ratio test for the rate of population decline of *Bacillus polymyxa* (Pw-2R) inoculated onto bare soil in both laboratory intact soil core microcosms and field plots.

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3$,
where Y: Log CFU/g fresh soil, μ_x: constants, and DAY: Days after inoculation.

(a) Field

Source of Variation	DF	SS
Regression	3	46.76593
Residual	24	11.43754
Total	27	

(b) Lab

Source of Variation	DF	SS
Regression	3	38.24962
Residual	22	7.498513
Total	25	

$SS_{Res(max)} = SS_{Res(Field)} + SS_{Res(Lab)} = 18.93605$
$DF_{Res(max)} = DF_{Res(Field)} + DF_{Res(Lab)} = 4$

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(c) Parallel Model = Field +Lab + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	4	85.92189
Residual	49	19.30014
Total	53	

Test: H_o: the regression equations describe parallel surfaces.
H_1: the regression equations do not describe parallel surfaces.

$$F_{Test} = \frac{\frac{SS_{Res(par)} - SS_{Res(max)}}{DF_{Res(par)} - DF_{Res(max)}}}{\frac{SS_{Res(max)}}{DF_{Res(max)}}}$$

$F_{Test} = 0.29486$

$F_{Crit} = F_{0.05\,(3,46)} = 2.79$ $F_{Crit} >> F_{Test}$: The lines ARE parallel at $p<0.05$.

Table A2. Analysis of variance and F-ratio test for the coincidence of population decline of *Bacillus polymyxa* (Pw-2R) inoculated onto bare soil in both laboratory intact soil core microcosms and field plots.

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3$,
where Y: Log CFU/g fresh soil, μ_x: constants, and DAY: Days after inoculation.

(a) Coincidental Model = Field +Lab

Source of Variation	$DF_{(com)}$	$SS_{(com)}$
Regression	3	85.36295
Residual	50	19.85907
Total	53	

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(b) Parallel Model = Field +Lab + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	4	85.92189
Residual	49	19.30014
Total	53	

Test: H'_o: the two surfaces coincide given that they are parallel.
H'_1: the two surfaces do not coincide given that they are parallel.

$$F_{Test} = \frac{\frac{SS_{Res(com)} - SS_{Res(par)}}{1}}{\frac{SS_{Res(par)}}{DF_{Res(par)}}}$$

$F_{Test} = 1.4189$

$F_{Crit} = F_{0.05\,(1,49)} = 4.03$ $F_{Crit} >> F_{Test}$: The lines ARE coincidental at $p<0.05$.

Table A3. Analysis of variance and F-ratio test for the rate of population decline of *Pseudomonas fluorescens* (Sw5-RN) inoculated onto bare soil in both laboratory intact soil core microcosms and field plots.

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3$,
where Y: Log CFU/g fresh soil, μ_x: constants, and DAY: Days after inoculation.

(a) Field

Source of Variation	DF	SS
Regression	3	46.58873
Residual	24	13.60805
Total	27	

(b) Lab

Source of Variation	DF	SS
Regression	3	34.01674
Residual	21	7.23766
Total	24	

$SS_{Res(max)} = SS_{Res(Field)} + SS_{Res(Lab)} = 20.84571$
$DF_{Res(max)} = DF_{Res(Field)} + DF_{Res(Lab)} = 45$

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(c) Parallel Model = Field +Lab + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	4	80.36804
Residual	48	21.61826
Total	52	

Test: H_o: the regression equations describe parallel surfaces.
H_1: the regression equations do not describe parallel surfaces.

$$F_{Test} = \frac{\frac{SS_{Res(par)} - SS_{Res(max)}}{DF_{Res(par)} - DF_{Res(max)}}}{\frac{SS_{Res(max)}}{DF_{Res(max)}}}$$

$F_{Test} = 0.55591$

$F_{Crit} = F_{0.05\,(3,45)} = 2.81$ $F_{Crit} >> F_{Test}$: The lines ARE parallel at $p<0.05$.

Table A4. Analysis of variance and F-ratio test for the coincidence of population decline of *Pseudomonas fluorescens* (Sw5-RN) inoculated onto bare soil in both laboratory intact soil core microcosms and field plots.

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3$,
where Y: Log CFU/g fresh soil, μ_x: constants, and DAY: Days after inoculation.

(a) Coincidental Model = Field +Lab

Source of Variation	$DF_{(com)}$	$SS_{(com)}$
Regression	3	80.3293
Residual	49	21.657
Total	52	

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(b) Parallel Model = Field +Lab + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	4	80.36804
Residual	48	21.61826
Total	52	

Test: H'_o: the two surfaces coincide given that they are parallel.
H'_1: the two surfaces do not coincide given that they are parallel.

$$F_{Test} = \frac{\frac{SS_{Res(com)} - SS_{Res(par)}}{1}}{\frac{SS_{Res(par)}}{DF_{Res(par)}}}$$

$F_{Test} = 0.08602$

$F_{Crit} = F_{0.05\,(1,44)} = 4.02$ $F_{Crit} >> F_{Test}$: The lines ARE coincidental at $p<0.05$.

Table A5. Analysis of variance and F-ratio test for the rate of population decline of *Bacillus polymyxa* (Pw-2R) inoculated into the rhizosphere of one year old hybrid spruce seedlings in both laboratory intact soil core microcosms and field plots.

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3$,
where Y: Log CFU/g fresh root, μ_x: constants, and DAY: Days after inoculation.

(a) Field

Source of Variation	DF	SS
Regression	3	188.0498
Residual	81	44.00217
Total	84	

(b) Lab

Source of Variation	DF	SS
Regression	3	127.130
Residual	77	72.58817
Total	80	

$SS_{Res(max)} = SS_{Res(Field)} + SS_{Res(Lab)} = 116.59$
$DF_{Res(max)} = DF_{Res(Field)} + DF_{Res(Lab)} = 158$

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(c) Parallel Model = Field +Lab + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	4	315.579
Residual	161	123.077
Total	165	

Test: H_o: the regression equations describe parallel surfaces.
H_1: the regression equations do not describe parallel surfaces.

$$F_{Test} = \frac{\frac{SS_{Res(par)} - SS_{Res(max)}}{DF_{Res(par)} - DF_{Res(max)}}}{\frac{SS_{Res(max)}}{DF_{Res(max)}}}$$

$F_{Test} = 2.78$

$F_{Crit} = F_{0.05\,(3,158)} = 2.66$ $F_{Crit} < F_{Test}$: The lines are NOT parallel at $p<0.05$.
$F_{Crit} = F_{0.01\,(3,158)} = 3.78$ $F_{Crit} > F_{Test}$: The lines ARE parallel at $p<0.01$.

Table A6. Analysis of variance and F-ratio test for the coincidence of population decline of *Bacillus polymyxa* (Pw-2R) inoculated into the rhizosphere of one year old hybrid spruce seedlings in both laboratory intact soil core microcosms and field plots.

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3$,
where Y: Log CFU/g fresh root, μ_x: constants, and DAY: Days after inoculation.

(a) Coincidental Model = Field +Lab

Source of Variation	$DF_{(com)}$	$SS_{(com)}$
Regression	3	307.369
Residual	162	131.2874
Total	165	

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(b) Parallel Model = Field +Lab + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	4	315.579
Residual	161	123.077
Total	165	

Test: H'_o: the two surfaces coincide given that they are parallel.
H'_1: the two surfaces do not coincide given that they are parallel.

$$F_{Test} = \frac{\frac{SS_{Res(com)} - SS_{Res(par)}}{1}}{\frac{SS_{Res(par)}}{DF_{Res(par)}}}$$

$F_{Test} = 10.74$

$F_{Crit} = F_{0.05\,(1,162)} = 3.9$ $F_{Crit} << F_{Test}$: The lines are NOT coincidental at $p<0.05$.
$F_{Crit} = F_{0.01\,(1,162)} = 6.82$ $F_{Crit} << F_{Test}$: The lines are NOT coincidental at $p<0.01$.

Table A7. Analysis of variance and F-ratio test for the rate of population decline of *Pseudomonas fluorescens* (Sw5-RN) inoculated into the rhizosphere of one year old hybrid spruce seedlings in both laboratory intact soil core microcosms and field plots.

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3$,
where Y: Log CFU/g fresh root, μ_x: constants, and DAY: Days after inoculation.

(a) Field

Source of Variation	DF	SS
Regression	3	216.7127
Residual	82	64.03867
Total	85	

(b) Lab

Source of Variation	DF	SS
Regression	3	199.4587
Residual	77	58.82832
Total	80	

$SS_{Res(max)} = SS_{Res(Field)} + SS_{Res(Lab)} = 122.86699$
$DF_{Res(max)} = DF_{Res(Field)} + DF_{Res(Lab)} = 159$

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(c) Parallel Model = Field +Lab + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	4	411.809
Residual	167	127.40688
Total	171	

Test: H_o: the regression equations describe parallel surfaces.
H_1: the regression equations do not describe parallel surfaces.

$$F_{Test} = \frac{\frac{SS_{Res(par)} - SS_{Res(max)}}{DF_{Res(par)} - DF_{Res(max)}}}{\frac{SS_{Res(max)}}{DF_{Res(max)}}}$$

$F_{Test} = 2.01991$

$F_{Crit} = F_{0.05\,(3,164)} = 2.60$ $F_{Crit} > F_{Test}$: The lines ARE parallel at $p<0.05$.

Table A8. Analysis of variance and F-ratio test for the coincidence of population decline of *Pseudomonas fluorescens* (Sw5-RN) inoculated into the rhizosphere of one year old hybrid spruce seedlings in both laboratory intact soil core microcosms and field plots.

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3$,
where Y: Log CFU/g fresh root, μ_x: constants, and DAY: Days after inoculation.

(a) Coincidental Model = Field +Lab

Source of Variation	$DF_{(com)}$	$SS_{(com)}$
Regression	3	411.47745
Residual	168	127.7387
Total	171	

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(b) Parallel Model = Field +Lab + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	4	411.809
Residual	167	127.40688
Total	171	

Test: H'_0: the two surfaces coincide given that they are parallel.
H'_1: the two surfaces do not coincide given that they are parallel.

$$F_{Test} = \frac{\frac{SS_{Res(com)} - SS_{Res(par)}}{1}}{\frac{SS_{Res(par)}}{DF_{Res(par)}}}$$

$F_{Test} = 0.43494$

$F_{Crit} = F_{0.05\,(1,167)} = 3.84$ $F_{Crit} << F_{Test}$: The lines ARE coincidental at $p<0.05$.

Table A9. Analysis of variance and F-ratio test for the rate of total biomass accumulation of one year old hybrid spruce seedlings planted in field plots *and* inoculated with *Pseudomonas fluorescens* (Sw5-RN) and *Bacillus polymyxa* (Pw-2R).

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY$,
where Y: Log total dry weight(g), μ_x: constants, and DAY: Days after inoculation.

(a) Field Pw-2R

Source of Variation	DF	SS
Regression	1	16.26731
Residual	113	2.61954
Total	114	

(b) Field Sw5-RN

Source of Variation	DF	SS
Regression	1	16.80608
Residual	111	2.22862
Total	112	

$SS_{Res(max)} = SS_{Res(Field)} + SS_{Res(Lab)} = 4.84816$
$DF_{Res(max)} = DF_{Res(Field)} + DF_{Res(Lab)} = 224$

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(c) Parallel Model = Field Pw-2R + Field Sw5-RN + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	2	33.06628
Residual	225	4.85822
Total	227	

Test: H_o: the regression equations describe parallel surfaces.
H_1: the regression equations do not describe parallel surfaces.

$$F_{Test} = \frac{\frac{SS_{Res(par)} - SS_{Res(max)}}{DF_{Res(par)} - DF_{Res(max)}}}{\frac{SS_{Res(max)}}{DF_{Res(max)}}}$$

$F_{Test} = 0.46480$

$F_{Crit} = F_{0.05\,(1,224)} = 3.87$ $F_{Crit} >> F_{Test}$: The lines ARE parallel at $p<0.05$.

Table A10. Analysis of variance and F-ratio test for coincidence of total biomass accumulation of one year old hybrid spruce seedlings planted in field plots *and* inoculated with *Pseudomonas fluorescens* (Sw5-RN) and *Bacillus polymyxa* (Pw-2R).

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY$,
where Y: Log total dry weight (g), μ_x: constants, and DAY: Days after inoculation.

(a) Coincidental Model = Field Pw-2R +Field Sw5-RN

Source of Variation	$DF_{(com)}$	$SS_{(com)}$
Regression	1	33.04919
Residual	226	4.87531
Total	227	

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(b) Parallel Model = Field Pw-2R +Field Sw5-RN + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	2	33.06628
Residual	225	4.85822
Total	227	

Test: H'_o: the two surfaces coincide given that they are parallel.
H'_1: the two surfaces do not coincide given that they are parallel.

$$F_{Test} = \frac{\frac{SS_{Res(com)} - SS_{Res(par)}}{1}}{\frac{SS_{Res(par)}}{DF_{Res(par)}}}$$

$F_{Test} = 0.79149$

$F_{Crit} = F_{0.05\,(1,225)} = 3.87$ $F_{Crit} << F_{Test}$: The lines ARE coincidental at $p<0.05$.

Table A11. Analysis of variance and F-ratio test for the rate of total biomass accumulation of one year old hybrid spruce seedlings planted in field plots and left uninoculated (Control) compared with those inoculated with *Bacillus polymyxa* (Pw-2R).

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY$,
where Y: Log total dry weight(g), μ_x: constants, and DAY: Days after inoculation.

(a) Field Pw-2R

Source of Variation	DF	SS
Regression	1	16.26731
Residual	113	2.61954
Total	114	

(b) Field Control

Source of Variation	DF	SS
Regression	1	5.97272
Residual	46	1.38692
Total	47	

$SS_{Res(max)} = SS_{Res(Field)} + SS_{Res(Lab)} = 4.00646$
$DF_{Res(max)} = DF_{Res(Field)} + DF_{Res(Lab)} = 159$

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(c) Parallel Model = Field Pw-2R + Field Control + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	2	22.6899
Residual	160	4.0077
Total	162	

Test: H_o: the regression equations describe parallel surfaces.
H_1: the regression equations do not describe parallel surfaces.

$$F_{Test} = \frac{\frac{SS_{Res(par)} - SS_{Res(max)}}{DF_{Res(par)} - DF_{Res(max)}}}{\frac{SS_{Res(max)}}{DF_{Res(max)}}}$$

$F_{Test} = 0.04921$

$F_{Crit} = F_{0.05\ (1,159)} = 3.90$ $F_{Crit} >> F_{Test}$: The lines ARE parallel at $p<0.05$.

Table A12. Analysis of variance and F-ratio test for coincidence of total biomass accumulation of one year old hybrid spruce seedlings planted in field plots *and* inoculated with *Pseudomonas fluorescens* (Sw5-RN) and *Bacillus polymyxa* (Pw-2R).

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY$,
where Y: Log total dry weight (g), μ_x: constants, and DAY: Days after inoculation.

(a) Coincidental Model = Field Pw-2R +Field Control

Source of Variation	$DF_{(com)}$	$SS_{(com)}$
Regression	1	22.68311
Residual	161	4.01499
Total	162	

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(b) Parallel Model = Field Pw-2R + Field Control + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	2	22.6899
Residual	160	4.0077
Total	162	

Test: H'_o: the two surfaces coincide given that they are parallel.
H'_1: the two surfaces do not coincide given that they are parallel.

$$F_{Test} = \frac{\frac{SS_{Res(com)} - SS_{Res(par)}}{1}}{\frac{SS_{Res(par)}}{DF_{Res(par)}}}$$

$F_{Test} = 0.29104$

$F_{Crit} = F_{0.05\,(1,160)} = 3.90$ $F_{Crit} << F_{Test}$: The lines ARE coincidental at $p<0.05$.

Table A13. Analysis of variance and F-ratio test for the rate of total biomass accumulation of one year old hybrid spruce seedlings planted in field plots and left uninoculated (Control) compared with those inoculated with *Pseudomonas fluorescens* (Sw5-RN).

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY$,
where Y: Log total dry weight(g), μ_x: constants, and DAY: Days after inoculation.

(a) Field Sw5-RN

Source of Variation	DF	SS
Regression	1	16.80608
Residual	111	2.22862
Total	112	

(b) Field Control

Source of Variation	DF	SS
Regression	1	5.97272
Residual	46	1.38692
Total	47	

$SS_{Res(max)} = SS_{Res(Field)} + SS_{Res(Lab)} = 3.61554$
$DF_{Res(max)} = DF_{Res(Field)} + DF_{Res(Lab)} = 157$

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(c) Parallel Model = Field Sw5-RN + Field Control + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	2	23.28379
Residual	158	3.61698
Total	160	

Test: H_o: the regression equations describe parallel surfaces.
H_1: the regression equations do not describe parallel surfaces.

$$F_{Test} = \frac{\frac{SS_{Res(par)} - SS_{Res(max)}}{DF_{Res(par)} - DF_{Res(max)}}}{\frac{SS_{Res(max)}}{DF_{Res(max)}}}$$

$F_{Test} = 0.06253$

$F_{Crit} = F_{0.05\,(1,157)} = 3.90$ $F_{Crit} >> F_{Test}$: The lines ARE parallel at $p<0.05$.

Table A14. Analysis of variance and F-ratio test for coincidence of total biomass accumulation of one year old hybrid spruce seedlings planted in field plots and left uninoculated (Control) compared with those inoculated with *Pseudomonas fluorescens* (Sw5-RN).

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY$,
where Y: Log total dry weight (g), μ_x: constants, and DAY: Days after inoculation.

(a) Coincidental Model = Field Sw5-RN +Field Control

Source of Variation	$DF_{(com)}$	$SS_{(com)}$
Regression	1	23.28378
Residual	159	3.616977
Total	160	

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(b) Parallel Model = Field Sw5-RN + Field Control + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	2	23.28379
Residual	158	3.616975
Total	160	

Test: H'_o: the two surfaces coincide given that they are parallel.
H'_1: the two surfaces do not coincide given that they are parallel.

$$F_{Test} = \frac{\frac{SS_{Res(com)} - SS_{Res(par)}}{1}}{\frac{SS_{Res(par)}}{DF_{Res(par)}}}$$

$F_{Test} = 0.00009$

$F_{Crit} = F_{0.05\,(1,158)} = 3.90$ $F_{Crit} << F_{Test}$: The lines ARE coincidental at $p<0.05$.

Table A15. Analysis of variance and F-ratio test for the rate of total biomass accumulation of one year old hybrid spruce seedlings planted in laboratory intact soil core microcosms and inoculated with *Pseudomonas fluorescens* (Sw5-RN) and *Bacillus polymyxa* (Pw-2R).

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY$,
where Y: Log total dry weight(g), μ_x: constants, and DAY: Days after inoculation.

(a) Lab Pw-2R

Source of Variation	DF	SS
Regression	1	4.17941
Residual	89	0.98631
Total	90	

(b) Lab Sw5-RN

Source of Variation	DF	SS
Regression	1	5.64283
Residual	110	1.19738
Total	111	

$SS_{Res(max)} = SS_{Res(Field)} + SS_{Res(Lab)} = 2.18369$
$DF_{Res(max)} = DF_{Res(Field)} + DF_{Res(Lab)} = 199$

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(c) Parallel Model = Lab Pw-2R + Lab Sw5-RN + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	2	10.13279
Residual	200	2.18454
Total	202	

Test: H_o: the regression equations describe parallel surfaces.
H_1: the regression equations do not describe parallel surfaces.

$$F_{Test} = \frac{\frac{SS_{Res(par)} - SS_{Res(max)}}{DF_{Res(par)} - DF_{Res(max)}}}{\frac{SS_{Res(max)}}{DF_{Res(max)}}}$$

$F_{Test} = 0.07746$

$F_{Crit} = F_{0.05\ (1,199)} = 3.90$ $F_{Crit} >> F_{Test}$: The lines ARE parallel at $p<0.05$.

Table A16. Analysis of variance and F-ratio test for coincidence of total biomass accumulation of one year old hybrid spruce planted in laboratory intact soil core microcosms and inoculated with *Pseudomonas fluorescens* (Sw5-RN) and *Bacillus polymyxa* (Pw-2R).

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY$,
where Y: Log total dry weight (g), μ_x: constants, and DAY: Days after inoculation.

(a) Coincidental Model = Lab Pw-2R +Lab Sw5-RN

Source of Variation	$DF_{(com)}$	$SS_{(com)}$
Regression	1	10.10438
Residual	201	2.21295
Total	202	

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(b) Parallel Model = Lab Pw-2R + Lab Sw5-RN + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	2	10.13279
Residual	200	2.18454
Total	202	

Test: H'_o: the two surfaces coincide given that they are parallel.
H'_1: the two surfaces do not coincide given that they are parallel.

$$F_{Test} = \frac{\frac{SS_{Res(com)} - SS_{Res(par)}}{1}}{\frac{SS_{Res(par)}}{DF_{Res(par)}}}$$

$F_{Test} = 2.60101$

$F_{Crit} = F_{0.05\,(1,200)} = 3.87$ $F_{Crit} << F_{Test}$: The lines ARE coincidental at $p<0.05$.

Table A17. Analysis of variance and F-ratio test for the rate of total biomass accumulation of one year old hybrid spruce seedlings planted in laboratory intact soil core microcosms and left uninoculated (Control) compared with those inoculated with *Bacillus polymyxa* (Pw-2R).

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY$,
where Y: Log total dry weight(g), μ_x: constants, and DAY: Days after inoculation.

(a) Lab Pw-2R

Source of Variation	DF	SS
Regression	1	4.17941
Residual	89	0.98631
Total	90	

(b) Lab Control

Source of Variation	DF	SS
Regression	1	1.41990
Residual	27	0.20837
Total	28	

$SS_{Res(max)} = SS_{Res(Field)} + SS_{Res(Lab)} = 1.19468$
$DF_{Res(max)} = DF_{Res(Field)} + DF_{Res(Lab)} = 116$

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(c) Parallel Model = Lab Pw-2R + Lab Control + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	2	5.61536
Residual	117	1.19528
Total	119	

Test: H_o: the regression equations describe parallel surfaces.
H_1: the regression equations do not describe parallel surfaces.

$$F_{Test} = \frac{\frac{SS_{Res(par)} - SS_{Res(max)}}{DF_{Res(par)} - DF_{Res(max)}}}{\frac{SS_{Res(max)}}{DF_{Res(max)}}}$$

$F_{Test} = 0.05826$

$F_{Crit} = F_{0.05\,(1,116)} = 3.94$ $F_{Crit} >> F_{Test}$: The lines ARE parallel at $p<0.05$.

Table A18. Analysis of variance and F-ratio test for coincidence of total biomass accumulation of one year old hybrid spruce planted in laboratory intact soil core microcosms and left uninoculated (Control) compared with those inoculated with *Bacillus polymyxa* (Pw-2R).

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY$,

where Y: Log total dry weight (g), μ_x: constants, and DAY: Days after inoculation.

(a) Coincidental Model = Lab Pw-2R +Lab Control

Source of Variation	$DF_{(com)}$	$SS_{(com)}$
Regression	1	5.60369
Residual	118	1.20694
Total	119	

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$

where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(b) Parallel Model = Lab Pw-2R + Lab Control + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	2	5.61536
Residual	117	1.19528
Total	119	

Test: H'_o: the two surfaces coincide given that they are parallel.

H'_1: the two surfaces do not coincide given that they are parallel.

$$F_{Test} = \frac{\frac{SS_{Res(com)} - SS_{Res(par)}}{1}}{\frac{SS_{Res(par)}}{DF_{Res(par)}}}$$

$F_{Test} = 1.14232$

$F_{Crit} = F_{0.05\,(1,117)} = 3.92$ $F_{Crit} < F_{Test}$: The lines ARE coincidental at $p<0.05$.

Table A19. Analysis of variance and F-ratio test for the rate of total biomass accumulation of one year old hybrid spruce seedlings planted in laboratory intact soil core microcosms and left uninoculated (Control) compared with those inoculated with *Pseudomonas fluorescens* (Sw5-RN).

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY$,
where Y: Log total dry weight(g), μ_x: constants, and DAY: Days after inoculation.

(a) Lab Sw5-RN

Source of Variation	DF	SS
Regression	1	5.64283
Residual	110	1.19738
Total	111	

(b) Lab Control

Source of Variation	DF	SS
Regression	1	1.41990
Residual	27	0.20837
Total	28	

$SS_{Res(max)} = SS_{Res(Field)} + SS_{Res(Lab)} = 1.40574$
$DF_{Res(max)} = DF_{Res(Field)} + DF_{Res(Lab)} = 137$

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(c) Parallel Model = Lab Sw5-RN + Lab Control + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	2	7.32284
Residual	138	1.40577
Total	140	

Test: H_o: the regression equations describe parallel surfaces.
H_1: the regression equations do not describe parallel surfaces.

$$F_{Test} = \frac{\frac{SS_{Res(par)} - SS_{Res(max)}}{DF_{Res(par)} - DF_{Res(max)}}}{\frac{SS_{Res(max)}}{DF_{Res(max)}}}$$

$F_{Test} = 0.00292$

$F_{Crit} = F_{0.05\ (1,137)} = 3.91$ $F_{Crit} >> F_{Test}$: The lines ARE parallel at $p<0.05$.

Table A20. Analysis of variance and F-ratio test for coincidence of total biomass accumulation of one year old hybrid spruce seedlings planted in laboratory intact soil core microcosms and left uninoculated (Control) compared with those inoculated with *Pseudomonas fluorescens* (Sw5-RN).

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY$,
where Y: Log total dry weight (g), μ_x: constants, and DAY: Days after inoculation.

(a) Coincidental Model = Lab Sw5-RN +Lab Control

Source of Variation	$DF_{(com)}$	$SS_{(com)}$
Regression	1	7.27134
Residual	139	1.45727
Total	140	

ANOVA MODEL: $Y = \mu_0 + \mu_1 DAY + \mu_2 DAY^2 + \mu_3 DAY^3 + \mu_4 Dummy$
where Y: Log CFU/g fresh soil, μ_x: constants, DAY: Days after inoculation, and Dummy: Dummy variable.

(b) Parallel Model = Lab Sw5-RN + Lab Control + Dummy

Source of Variation	$DF_{(par)}$	$SS_{(par)}$
Regression	2	7.32284
Residual	138	1.40577
Total	140	

Test: H'_o: the two surfaces coincide given that they are parallel.
H'_1: the two surfaces do not coincide given that they are parallel.

$$F_{Test} = \frac{\frac{SS_{Res(com)} - SS_{Res(par)}}{1}}{\frac{SS_{Res(par)}}{DF_{Res(par)}}}$$

$F_{Test} = 5.05559$

$F_{Crit} = F_{0.05\ (1,138)} = 3.91$ $F_{Crit} << F_{Test}$: The lines ARE NOT coincidental at $p<0.05$.
$F_{Crit} = F_{0.01\ (1,138)} = 6.83$ $F_{Crit} > F_{Test}$: The lines ARE coincidental at $p<0.01$.