

**Characterization of root colonization by the biocontrol bacterium *Bacillus firmus* strain GB126.**

by

Mark Lee Durham

A thesis submitted to the Graduate Faculty of  
Auburn University  
in partial fulfillment of the  
requirements for the Degree of  
Master of Science

Auburn, Alabama  
August 3, 2013

Keywords: Root colonization, *Bacillus firmus*, PGPR, root printing, dilution plating

Copyright 2013 by Mark Lee Durham

Approved by

Joseph W. Kloepper, Chair, Professor of Plant Pathology  
Kathy S. Lawrence, Professor of Plant Pathology  
Mark R. Liles, Associate Professor of Biological Sciences

## Abstract

A new biological control treatment for plant-parasitic nematodes was marketed throughout the U.S. starting in 2010. The treatment consists of the biocontrol agent *Bacillus firmus* strain GB126. Following commercial release of the treatment, questions arose about the persistence of GB126 during the growing season. The work reported in this thesis was designed to address this key question by developing techniques to monitor root colonization of GB126. To accomplish this, root printing and dilution plating techniques were developed to monitor root colonization of GB126. Results showed that GB126 colonized the roots of several plant species and colonized soybean in soils ranging from pH 5.05 to 7.05. More importantly, GB126 was isolated from the rhizoplane and endorhiza of plant roots at extended periods after seed inoculation. The results of this study show that GB126 can persist externally and internally on roots following application as a seed treatment.

## Acknowledgments

The author would like to express his appreciation to Bayer CropScience for its financial support in this research. Special thanks must be given to Dr. Joseph Kloepper for his guidance, encouragement, and dedication my professional development and to this project. Thanks also goes to the committee members, Dr. Kathy Lawrence and Dr. Mark Liles, for their insight and discussions. The author wishes to thank his family and friends for their love and support. Finally, the author would like to extend his gratitude to his fellow graduate students and faculty members for their contributions in a variety of ways.

## Table of Contents

Abstract .....	ii
Acknowledgments.....	iii
List of Tables .....	v
List of Figures .....	vi
List of Abbreviations .....	viii
Literature Review .....	1
Materials and Methods .....	30
Results .....	40
Assessing Sporulation of Rifampicin-Resistant GB126 Mutants	
Optimization of Root Printing	
Dilution Plating for Quantifying External and Internal Root Colonization	
Comparative Colonization of Soybean in Acidic Soils	
Microplot Trials to Confirm Colonization Capacity	
Discussion .....	46
References.....	57
Tables .....	61
Figures .....	65

## List of Tables

Table 1. Population densities of GB126 rif at 6 WAP corn, cotton, and soybean. ....	61
Table 2. Populaiton densities of GB126 rif at 8 WAP on soybean. ....	61
Table 3. Population densities of GB126 rif at 10 WAP on corn. ....	62
Table 4. Population densities of GB126 rif at 10 WAP on cotton. ....	62
Table 5. Population densities of GB126 rif at 12 WAP on corn. ....	63
Table 6. Population densities of GB126 rif at 12 WAP on cotton. ....	63
Table 7. Population densities of GB126 rif at 12 WAP on soybean. ....	64

## List of Figures

- Figure 1. Light microscope images of root cross-sections of *Miscanthus x giganteus* plant inoculated with *Herbaspirillum frisingense* Mb11. Rothballer et al. (2008). ..... 20
- Figure 2. Microphotographs of rhizoplane and endorhiza of grape plantlets after inoculation with *Burkholderia* sp. strain PsJN. Compant et al. (2005). ..... 25
- Figure 3. Images of the fifth internode and leaf internal tissues of grape plantlets after rhizosphere inoculation of *Burkholderia* sp. strain PsJN. Compant et al. (2005). .... 27
- Figure 4. Typical colony morphology of *Bacillus firmus* strain GB126 rif on 50% TSArif, pH 8.0. .... 65
- Figure 5. Root printing of soybean (left) and cotton (right) root sections onto 50% TSArif without fungicides at 2 WAP. .... 65
- Figure 6. Root printing of corn (upper), cotton (middle), and soybean (lower) root sections on 50% TSA rif with fungicides showing growth of GB126 colonies (left plates). ..... 66
- Figure 7. Root printing of soybean roots in semi-solid agar from the “control” soil (upper plates) and Blackbelt soil (lower plates) at 14 DAP. .... 67
- Figure 8. Root printing of soybean roots in semi-solid agar from the Piedmont soil (upper plates) and Sand Mountain soil (lower plates) at 14 DAP. .... 68
- Figure 9. Root printing of soybean roots in semi-solid agar from the Wiregrass soil at 14 DAP. . ..... 69
- Figure 10. Average log CFU/g populations of GB126 rif at 3, 6, and 12 WAP on corn from microplot trial 1..... 69
- Figure 11. Average log CFU/g populations of GB126 rif at 3, 6, and 12 WAP on cotton from microplot trial 1..... 70
- Figure 12. Average log CFU/g populations of GB126 rif at 3, 6, and 12 WAP on soybean from microplot trial 1..... 70
- Figure 13. Average log CFU/g populations of GB126 rif at 3, 6, and 12 WAP on soybean from microplot trial 2 using a 3:1 ratio of soil from E.V. Smith to sand. .... 71

Figure 14. Average log CFU/g populations of GB126 rif at 3, 6, and 12 WAP on soybean from microplot trial 2 using Blackbelt soil type..... 71

Figure 15. Average log CFU/g populations of GB126 rif at 3, 6, and 12 WAP on soybean from microplot trial 2 using Piedmont soil type..... 72

Figure 16. Average log CFU/g populations of GB126 rif at 3, 6, and 12 WAP on soybean from microplot trial 2 using Sand Mountain soil type..... 72

Figure 17. Average log CFU/g populations of GB126 rif at 3, 6, and 12 WAP on soybean from microplot trial 2 using Wiregrass soil type. .... 73

## List of Abbreviations

rif	Rifampicin
CFU	Colony Forming Units
DAI	Days After Inoculation
PSRC	Plant Science Research Center
WAP	Weeks After Planting
DAP	Days After Planting
ISR	Induced Systemic Resistance

## II. LITERATURE REVIEW

Modern agriculture has enabled farmers to achieve crop yields capable of providing food for an ever growing population worldwide. Numerous technologies are being investigated and employed to augment conventional agriculture. One such area of research is the field of biological control. Biological control can be defined as the total or partial inhibition or destruction of pathogen populations by other organisms (Agrios 2005). There are a vast number of organisms under investigation that could act as biological control agents, and bacteria are of major interest. Bacteria belonging to the genera *Acetobacter*, *Acinebacter*, *Alcaligenes*, *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Derxia*, *Enterobacter*, *Gluconacetobacter*, *Herbaspirillum*, *Klebsiella*, *Ochrobacterum*, *Pantoea*, *Pseudomonas*, *Rhodococcus*, *Serratia*, *Stenotrophomonas*, and *Zoogloea* have been the subject of extensive research concerning biological control (Babalola 2010). Research on plant-microbe interactions that promote plant health and plant development have often been focused on rhizosphere bacteria. These rhizobacteria are associated with all the nearly 300,000 existing plant species on the earth, and some of these rhizobacteria are endophytes (Ryan et al. 2008). Only a few plant species have been carefully studied relative to the biology of plant-bacteria interactions. There remains a considerable amount of unknown information about potential bacteria that may possess beneficial plant-host relationships.

## **Plant Growth-Promoting Bacteria**

Of the bacteria that are known to interact with plants are bacteria known as plant growth-promoting rhizobacteria (PGPR). PGPR are specific strains of rhizosphere bacteria that stimulate plant growth (Kloepper and Schroth 1978). Such bacteria are also sometimes referred to as “plant growth-promoting bacteria” or simply PGPB (Bashan and Holguin 1998). In either case, plant growth-promoting rhizobacteria offer benefits to their host plant. The term PGPR is more preferred and will be used in this paper since it is encountered more in literature and focuses on bacteria associated with plant rhizospheres. Another, more precise definition of PGPR is that these bacteria are non-pathogenic, strongly root colonizing bacteria on the surface of a plant’s roots, which increase a plant’s yield by one or more mechanisms (Babalola 2010).

One term that is closely associated with PGPR is endophytes. The term “endophyte” is derived from the Greek ‘endon’ (within) and ‘phyte’ (plant). Until recently, the term was usually applied to fungi (Carroll 1988). However, a more appropriate definition of the term endophyte includes fungi or bacteria which for all or part of their life cycle invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues but cause no symptoms of disease (Sturz et al. 2000). While PGPR and endophytes are two different terms, many PGPR exhibit endophytic behavior. Considering this fact, the best definition to use for the term is endophyte which would include any bacterium that can be isolated from surface-disinfested plant tissue or extracted from inside the plant and that does not appear to visibly harm the plant host (Hallmann et al. 1997). This definition is more functional since it is inclusive of bacteria that, during their endophytic phase, may fluctuate between endophytic and epiphytic colonization.

## Benefits of Utilizing PGPR

Among the potential benefits offered by PGPR is a reduction in the amount of fertilizer needed to sustain agricultural systems. Fertilizers are essential in modern agriculture because they provide the nutrients plants need for growth and development. However, fertilizer use has negative effect on the environment if misused. One example of the negative impacts of fertilizer use is a “dead zone” in the Gulf of Mexico where nutrients washing away from farms across the Mississippi Basin cause oxygen starvation in the Gulf, resulting in an area in that is virtually lifeless (Malakoff 1998). PGPR offer a potential way to reduce dependence on chemical fertilizers. For example, a study conducted by Adesemoye et al. (2009) showed that using PGPR strains such as *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilis* T4 produced plant growth, enhanced yield, and increased nutrient uptake using 75% of the recommended fertilizer rate. This combination of PGPR strains and reduced fertilizer rate produced results that were statistically equivalent to using the full fertilizer rate without inoculants. If PGPR are capable of promoting plant growth with reduced fertilizer rates, it may be possible to utilize PGPR inoculants to reduce the dependence on chemical fertilizers.

PGPR also offer benefits in the form of plant growth promotion. Numerous mechanisms of promoting plant growth exist, and some PGPR may possess more than one mechanism (Ahmad et al. 2008). Among such mechanisms are siderophore production, antagonism to pathogenic fungi, nitrogen fixation, phosphate solubilization, the production of organic acids and indole acetic acid (IAA), ammonia (NH<sub>3</sub>), hydrogen cyanide (HCN), the release of enzymes (soil dehydrogenase, phosphatase, nitrogenase, etc.), and the induction of systemic disease resistance (ISR). Babalola (2010) conducted a review regarding PGPR, which provides a detailed discussion of plant growth-promoting mechanisms.

## **Regions of Bacterial Colonization**

Bacteria colonize plants in different areas or regions typically defined as the phylloplane, rhizosphere, rhizoplane, and endorhizal zones. The terms epiphytic and endophytic are also commonly used in defining colonization to refer broadly to colonization of the exterior and interior of the plant, respectively. Bacteria that colonize the phylloplane are capable of multiplying on plant surfaces such as the leaves and stems (Hirano and Upper 1983). Phylloplane-colonizing bacteria may colonize epiphytically and endophytically. The most common sites of colonization are the bases of trichomes (Timmer et al. 1987), stomata (Romanstchuk 1992), and epidermal cell wall junctions (Davis and Brlansky 1991), especially in the grooves along the leaf veins (Mariano and McCarter 1993). Phylloplane-colonizing bacteria need survival strategies in order to grow and reproduce because of environmental stresses on the leaf surface, such as low water availability and UV radiation. Hence, bacteria colonizing the phylloplane typically adopt one of two strategies: a tolerance strategy or an avoidance strategy. A tolerance strategy requires the ability to tolerate environmental conditions on the phylloplane, while with an avoidance strategy bacteria colonize sites which are more protected from environmental stresses (Beattie and Lindow 1995).

In contrast to the phylloplane, microorganisms associated with plant roots are located in three habitats: the soil, rhizosphere, and endorhiza. The soil habitat can be defined as being unaffected by plant root exudates or other material produced by living plant roots. The rhizosphere is generally defined as the zone of soil affected by root exudates and other material supplied by plant roots. However, considering that many methods used to sample the rhizosphere do not allow for complete separation of this zone of soil from the rhizoplane (plant root surface), a practical definition of the rhizosphere would be “the zone of soil influenced by plant-produced

material and the epidermal layer of the plant root” (Mahaffee and Kloepper 1997). The final term, endorhiza, refers to plant tissues beneath the epidermal layer, including cortical and vascular tissues (Mahaffee and Kloepper 1997).

When referring to any of the zones of colonization, bacteria are characterized as being epiphytic or endophytic regarding their location in the plant tissues. Epiphytic bacteria are generally defined as bacteria capable of living (i.e., multiplying) on plant surfaces (Hirano and Uppur 1983). In contrast, endophytic bacteria have been defined as “bacteria that live within living plant tissues without doing substantive harm or gaining benefit other than securing residency” (Bressan and Borges 2004). When considering epiphytic and endophytic colonization, some authors have defined the terms as “two ends of a spectrum reflecting the growth patterns of bacteria rather than as two distinct groups of organisms” (Beattie and Lindow 1995). However, this approach to defining epiphytic and endophytic bacteria is restrictive as it places bacteria into one of two groups, referring to internal and external populations of one bacterial strain. To differentiate epiphytic and endophytic bacteria more accurately, endophytic bacteria can be considered as “any bacterium that can be isolated from surface-disinfested plant tissue or extracted from inside the plant, and if it does not visibly harm the plant” (Hallmann et al. 1997). This definition includes internal colonists with apparently neutral behavior as well as symbionts. It also is inclusive of bacteria that, during their endophytic phase, fluctuate between epiphytic and endophytic colonization. Although these habitats may appear to be conceptually distinct, they are actually parts of a continuum where each habitat blends to the next (Mahaffee and Kloepper 1997). Evidence for this concept is seen in several studies comparing internal and external bacterial communities of cucumber (Mahaffee and Kloepper 1997), cotton (Hallmann et al. 1999), and potato (Sturz 1995) in which almost all endophytic bacteria were also found in the

rhizosphere. This finding suggests that root-colonizing microorganisms that are endophytic are likely to be found from the rhizosphere to rhizoplane, epidermis, and cortex (Kloepper et al. 1992). Thus, endophytic bacteria are most likely to also be epiphytic, while epiphytic bacteria are not necessarily endophytic.

When referring to colonization of plant roots, it is important to emphasize that colonization is not synonymous with growth on roots in a gnotobiotic system (i.e., soil free or sterile conditions). The concept of PGPR root colonization arose in the 1980s and was described as an active process that involves growth of the inoculated PGPR in the presence of native soil microorganisms (Kloepper et al. 1980). Using this definition of root colonization, one finds two elements are necessary for studying root colonization of PGPR. One, a marking system, such as fluorescence *in situ* hybridization (FISH) or green fluorescent protein (GFP), is needed in order to differentiate the introduced PGPR from native soil bacteria. Two, the plants must be grown in soil (non-sterile environment). These elements are important because they allow the consideration of native microorganisms that naturally occur in soils, which reflect practical applications of using PGPR. In recent years, studies have focused on root colonization involving different PGPR strains (Nautiyal 1997, Timmusk et al. 2005, and Buddrus-Scheimann et al. 2010). However, these studies do not meet the criteria mentioned above and thus only provide a partial understanding of root colonization since the methods utilized are essentially gnotobiotic in nature.

### **Review of Specific Means of Measuring Colonization, Case Studies**

There are many publications in which root colonization by PGPR has been monitored and reported. In the following section, a selection of these reports is presented. The aim is to present selected examples of how various marking systems have been employed to investigate

colonization of roots or endophytic colonization by PGPR. The methods used in each study are summarized in sufficient detail to discuss strengths and limitations of various approaches to measuring colonization.

### ***Biofilm Formation and Root Invasion by Paenibacillus polymyxa utilizing GFP***

#### **Overview of study**

Timmusk et al. (2005) investigated root colonization and biofilm formation by *Paenibacillus polymyxa* strains B1 and B2 on *Arabidopsis thaliana*. The method of observing colonization utilized *gfp*-tagged *P. polymyxa* using a plasmid-borne *gfp* gene. Using GFP to tag *P. polymyxa* fulfills the first element for successfully determining bacterial root-colonizing because it offers a method of differentiating the introduced bacterium from any native microorganisms present in the rhizosphere. However, the second element of determining PGPR root colonization, demonstrating colonization in the presence of native soil microbes, was not fulfilled because the study was conducted under gnotobiotic conditions.

#### **Details of Study**

*Arabidopsis thaliana* ecotype C24 was used to study *P. polymyxa* root colonization. Seeds of *A. thaliana* were first surface sterilized by incubating them in a saturated and filtered solution of aqueous calcium chlorate solution followed by repeated washes in sterile distilled water. The seeds were subsequently sown into Murashige & Skoog (MS)-2 medium for germination. After germinating, seedlings were transferred to new culture dishes containing MS medium and were allowed to grow for two weeks in a growth chamber. Once the *A. thaliana* seedlings were grown for two weeks, the plants were inoculated with *P. polymyxa* strains B1 and B2. This was performed by soaking their roots in 10-ml diluted cultures of *P. polymyxa* ( $\sim 10^6$  bacteria  $\text{ml}^{-1}$ ) overnight. After inoculating the roots overnight in the *P. polymyxa* culture, the

plants were transferred to a 10-fold diluted Luria-Bertani (L) medium, and the colonization pattern of the bacteria was monitored once per hour for the first 12 hours after inoculation. To detect and enumerate *P. polymyxa* on *A. thaliana* roots, individual roots were blended with 10 mM MgSO<sub>4</sub> for about 3 minutes. For endophytic enumeration, the plant roots were surface sterilized in a solution of 1% sodium hypochlorite, 0.1% sodium lauryl sulfate, and 0.2% Tween 20 in phosphate-buffered saline (PBS). Following surface disinfection, the roots were vortexed for 2 minutes followed by four washes with sterilized water. The roots were examined under a microscope to ensure that no fluorescent cells remained. Additionally, wash solutions from the last root rinse were cultured to determine the efficiency of the sterilization process. Finally, roots were macerated using a mortar and pestle, and the numbers of CFU were determined by plating preparations on glycine betaine (GB) medium containing erythromycin at 10 µg ml<sup>-1</sup> (Timusk et al 2005).

Utilizing the *gfp*-tagged *P. polymyxa* strains and fluorescence microscopy, colonization on the roots of *A. thaliana* was visualized. Colonization occurred preferentially in defined regions of the zones of elongation and differentiation in the primary plant roots. The first site of colonization was the zone of elongation at the root tip, where bacteria started to accumulate as micro-colonies. Within 2 hours of inoculation, a biofilm consisting of bacterial cells and a semitransparent material suggestive of an extracellular matrix formed (10<sup>6</sup> cell g roots<sup>-1</sup>). Closer inspection of the root tip at a higher magnification and counting of root-invading bacteria indicated that fluorescent bacteria entered intercellular spaces of the plant roots within the first few hours of inoculation. This pattern of root invasion was well expressed by 5 hours post-inoculation, and root-invading bacteria were estimated to be 10<sup>5</sup> cells g roots<sup>-1</sup> (Timusk et al 2005).

Hence, the results of this study by Timusk et al. (2005) suggest that the *P. polymyxa* strains colonized roots and formed biofilms on the roots of *A. thaliana*. However, because the study was conducted under gnotobiotic conditions, the colonization reported is the same as root colonization in the presence of indigenous soil microorganisms. Specifically in this test, the *A. thaliana* plants were sterilized and grown in MS-medium for 2 weeks before being inoculated with the PGPR strains of *P. polymyxa*. The inoculation process involved soaking the 2-week-old roots in cultures of *P. polymyxa* ( $\sim 10^6$  bacteria ml<sup>-1</sup>) overnight. After the overnight inoculation, the *A. thaliana* seedlings were grown in a 10-fold diluted L medium and observed for colonization. The procedure did not test whether bacteria were able to colonize plant roots in the presence of native microorganisms. The methods used are akin coating the roots with bacteria and then observing bacterial survival on the roots rather than assessing colonization. However, the GFP-tagging system did allow visualization of the test bacterium on the roots.

### ***Root Colonization by Pseudomonas sp. DSMZ 13134 Monitored using FISH***

#### **Overview of Study**

In another study investigating root-colonizing PGPR, Buddrus-Schiemann et al. (2010) observed the interactions of *Pseudomonas* sp. on barley (*Hordeum vulgare* L.) utilizing FISH. This study focused on *Pseudomonas* sp. strain DSMZ 13134, which is the biological control agent in the product Proradix<sup>®</sup> (Sourcon Padena GmbH & Co. KG). The experiment was conducted using non-sterile and axenically cultured barley seeds with and without inoculation of strain DSMZ 13134. These conditions would fulfill the requirement of using nonsterile systems for studying root colonization. However, problems with the specificity of the fluorescently labeled oligonucleotide probe forced the authors to study the root colonization characteristics of *Pseudomonas* sp. DSMZ 13134 under axenic conditions using FISH for visualization.

## Details of Study

For this study, strain DSMZ 13134 was cultivated at 30°C on King's B medium adjusted to a pH of 7.2. The plant material used was summer barley cultivar Barke. In the laboratory experiments, barley plants were cultivated in sealed glass tubes filled with 70 g of washed and autoclaved quartz sand and 10 ml of sterile MS medium. Inoculated barley seeds were introduced into the quartz sand and grown for up to 4 weeks. For greenhouse experiments, loamy soil with a pH of 6.3 from an agricultural field was sieved (<10 mm) and added to pots. Barley seeds were surface sterilized with 70% ethanol for 2 minutes, incubated in 2% sodium hypochlorite solution (6-14% Cl active) for 15 minutes, and washed six times using sterile deionized water. The seeds were then subsequently incubated in a solution containing 600 µg/ml penicillin and 250 µg/ml streptomycin for 30 minutes. After this incubation period, the seeds were transferred to nutrient broth (NB) medium and placed in the dark for 2 days at 30°C. Seedlings without visible contamination after this period were chosen for inoculation.

Inoculation of the barley seeds with strain DSMZ 13134 was performed using vacuum infiltration with Proradix<sup>®</sup>. A suspension containing  $8 \times 10^{10}$  CFU/kg seeds was sprayed onto non-sterile seeds, and a vacuum was applied. The seeds were then dried at 27°C for 4 hours. Alternatively, sterile and non-sterile seedlings were inoculated with strain DSMZ 13134 cells. An overnight culture of strain DSMZ 13134 was washed twice with 1 x PBS and diluted to a concentration of  $10^8$  cells per ml 1 x PBS. Non-sterile seeds were germinated on water agar plates at 30°C in the dark for 2 days. Seedlings with a root length of 1 to 2 cm were incubated in the bacterial suspension at room temperature for 2 hours.

The FISH-method was used to detect strain DSMZ 13134 cells on the rhizoplane of the barley plants. After 2 weeks of growth in quartz sand or agricultural soil under unsterile

conditions, the plants were harvested and the roots sampled by shaking loose soil or quartz sand particles, gently washing them in sterile 1 x PBS in Petri dishes, separating the shoots, and briefly drying them on sterile paper tissue. Whole root systems were fixed with 4% paraformaldehyde and 1 x PBS (3:1) at 4°C for 2 hours.

The roots were then washed twice with 1 x PBS and placed in an ethanol/1 x PBS mixture (1:1) for longer storage at -20°C. For hybridization, 2-cm root pieces were removed from all parts of the root. Hybridization was performed by using 2 ml reaction vials with 120 µl hybridization buffer containing a formamide concentration of 35% and 15 µl of the oligonucleotide probes.

After washing, the root pieces were transferred to glass slides, air-dried, embedded with Citifluor, and covered with a cover slip. The following 16S rRNA-targeting oligonucleotide probes were used: an equimolar mixture of EUB-338-I, EUB-338-II, and EUB-338-III to detect all microorganisms of the domain Bacteria, and PSE-225 (5'-CCGACCTAGGCTCATCTA-3') for the specific identification of *Pseudomonas* cells. The probes were synthesized and labeled with the fluorescent dyes Cy3, and Fluos. Root colonization was followed using a confocal laser scanning microscope equipped with two helium neon lasers for excitation of the fluorophores Cy3 and Cy5 at wavelengths of 543 and 633 nm, and an argon laser for excitation of Fluos at a wavelength of 488 nm.

Bacteria cells were localized using a 63x water immersion objective. Cy3- and Fluos-labeled oligonucleotide probes provided red and green fluorescent signals, respectively. The combination of EUB-338-mix-Fluos and PSE-225-Cy3 resulted in a yellow color. After binding of both probes to strain DSMZ 13134, the bacteria cells present could be identified by yellow fluorescence. To visualize strain DSMZ 13134 in the rhizoplane of barley, the fluorescence *in*

*situ* hybridization performed in this study relied on the oligonucleotide probe PSE-225, which can detect several *Pseudomonas* sp. On the root surface of seedlings treated with Proradix<sup>®</sup> by vacuum filtration, *Pseudomonas* cells were found in great numbers colonizing first the root hair zones of plants that were grown in the quartz sand system. However, besides other bacteria, pseudomonads could be visualized in the rhizoplane of non-sterile control plants that did not receive the Proradix<sup>®</sup> treatment. This indicates that pseudomonads originating from the seeds effectively colonized the roots of germinating plants.

Since other *Pseudomonas* spp. could be detected using FISH, the authors had to use barley grown in axenic conditions to effectively examine the colonization pattern of strain DSMZ 13134 specifically. Using the information obtained from FISH, the authors concluded that strain DSMZ 13134 could be localized on the root surface from the root base up to the tip. They also indicated colonization occurred strongly in the root hair zone. Particularly high densities could be detected on the surface of root hairs. The problem with the methodology used in this study involves characterizing PGPR colonization. Although FISH was used to identify the desired PGPR, the oligonucleotide probes that were used in identifying strain DSMZ 13134 also hybridized with other *Pseudomonas* spp. In experiments conducted using non-sterile seeds of barley, naturally occurring pseudomonad populations were present as identified by the PSE-255-Cy3 probe. These populations forced the authors to study the root colonization pattern of strain DSMZ 13134 by using axenic conditions in order to eliminate the possibility of other *Pseudomonas* spp. interfering with observations.

## ***Rhizosphere Competence of PGPR Determined by Antibiotic Resistance***

### **Overview of Study**

Another method for studying PGPR colonization involves the use of antibiotic resistant marked mutant strains of potential PGPR. One such study performed by Nautiyal (1997) utilized spontaneous rifampicin-resistant (Rif<sup>r</sup>) derivatives of *Pseudomonas* sp. NBRI9926 and *Rhizobium* sp. NBRI9513 to elucidate suppression of pathogenic fungi in chickpea (*Cicer arietinum* L.). The spontaneous Rif<sup>r</sup> mutants obtained were *Pseudomonas* sp. NBRI9926P3 and *Rhizobium* sp. NBRI9513R7, which exhibited similar growth rates as the wild-type strains. These Rif<sup>r</sup> mutants of the wild-type bacteria were selected for their ability to inhibit *Fusarium oxysporum* f.sp. *ciceri*, *Rhizoctonia bataticola*, and *Pythium* sp. Greenhouse results demonstrated that *Pseudomonas* sp. NBRI9926P3 possessed a superior biocontrol potential against these three fungi compared to *Rhizobium* sp. NBRI9513R7.

### **Details of Study**

To obtain rhizosphere-competent bacteria, 256 bacterial strains were isolated from roots of chickpea plants grown in a fungal disease-suppressive field. The roots were harvested for bacterial strains 4 weeks after the 5-month-old chickpea crop was harvested. Serial dilutions of the root homogenates were plated on to *Pseudomonas* isolation agar, NA, tryptone-glucose-yeast extract (TGY) agar and yeast extract-mannitol (YEM) agar with Congo red (1% only for solid medium). Bacteria representative of different morphological types present on these plates were selected and purified on minimal medium based of AT salts. Bacterial strains were tested for inhibition of *F. oxysporum* f.sp. *ciceri*, *R. bataticola*, and *Pythium* sp. on nutrient agar. After an incubation period of 2 days for *R. bataticola* and *Pythium* sp. and 5-7 days for *F. oxysporum* f.sp. *ciceri*, the presence of inhibition zones was recorded. From these fungal growth inhibition

assays, *Pseudomonas* sp. and *Rhizobium* sp. were selected due to their antibiosis against the three fungi.

In order to monitor the presence of these two bacteria in sterile and non-sterile soils, spontaneous rifampicin-resistant (Rif<sup>r</sup>) strains of *Pseudomonas* sp. were isolated on *Pseudomonas* isolation agar, and strains of *Rhizobium* sp. were isolated on YEM agar plates. Both agar types contained 100 µg rifampicin. Spontaneous Rif<sup>r</sup> strains that had growth rates comparable to the wild-type were selected for further studies. Rif<sup>r</sup> strains were serially diluted and plated onto agar plates containing 0, 25, 50, and 100 µg rifampicin/ml. *Pseudomonas* isolation agar and YEM plates were used for growing *Pseudomonas* sp. and *Rhizobium* sp., respectively. No significant differences in plate counts were observed in the different plates. Hence, the concentration of rifampicin used was 50 µg rifampicin/ml as this was sufficient to inhibit growth of other bacteria in non-sterilized soils and was used to recover Rif<sup>r</sup> strains from the rhizosphere.

Seed bacterization for this study was carried out using chickpea (*Cicer arietinum* L. cv. Radhey) seeds and a bacterial inoculum prepared by scraping 48-hour cells from AT plates. Prior to bacterization, chickpea seeds were surface-sterilized by gently shaking (80 rpm on a reciprocal shaker at 28°C) with 70% ethanol (5 minutes) and 20% bleach Chlorox (10 minutes), followed by three rinses in sterile Milli-Q water (MQW). After surface-sterilization, seeds were soaked in the bacterial suspension for 4 hours at 28°C on a reciprocal shaker at 100 rpm. Control seeds (non-bacterized) were soaked in 0.85% saline MQW washed from uninoculated AT plates. Bacterization levels from the seeds were determined by agitating four seeds from each treatment in 4 ml 0.85% saline MQW followed by serial dilution on AT agar plates containing 50 µg rifampicin/ml. Mean colony forming units (cfu) per seed were determined by averaging the

cfu/seed values of three populations in three replicates per treatment after 48 hour incubation of the plates at 28°C. Using this method of seed bacterization, final cell concentrations for *Pseudomonas* sp. NBRI9926P3 and *Rhizobium* sp. NBRI9513R7 were determined to be  $4 \times 10^6$  cells/seed and  $1.2 \times 10^6$  cells/seed, respectively.

To test the biocontrol potential of *Pseudomonas* sp. NBRI9926P3 and *Rhizobium* sp. NBRI9513R7, non-sterile field soil conducive to the development of *F. oxysporum* f.sp. *ciceri*, *R. bataticola*, and *Pythium* sp. was used under greenhouse conditions. The experiment was carried out in four sets of 30 chickpea seedlings each for both treated and non-treated seeds (control). In each set, 45-day-old seedlings were monitored with respect to the number of healthy and diseased seedlings (dead seedlings, stunting of shoot height, drooping of leaves, root decolorization), dry weight, shoot length, and root length. Data were recorded 45 days after planting. Treatment of chickpea seeds with *Pseudomonas* sp. NBRI9926P3 and *Rhizobium* sp. NBRI9513R7 resulted in increases in germination by 22 and 34%, in survival by 24 and 43%, in dry weight by 21 and 44%, in shoot length by 16 and 29%, and in root length by 16 and 34%, respectively, compared to non-bacterized seeds.

This study demonstrates the ability of antibiotic-resistant marked PGPR strains *Pseudomonas* sp. NBRI9926P3 and *Rhizobium* sp. NBRI9513R7 to suppress plant pathogenic fungi *F. oxysporum* f.sp. *ciceri*, *R. bataticola*, and *Pythium* sp. Once the chickpea seedlings were sampled at 45 days, populations of either *Pseudomonas* sp. NBRI9926P3 or *Rhizobium* sp. NBRI9513R7 could have been determined by using *Pseudomonas* isolation agar or YEM plates amended with rifampicin. This would have allowed the author to determine the initial cell concentration present at planting as well as cell concentrations 45 days after planting. It would also have provided some insight as to the population dynamics of the microbial community in

the rhizosphere of the inoculated, antibiotic-resistant-marked strains of bacteria being studied. However, even if populations of *Pseudomonas* sp. NBRI9926P3 and *Rhizobium* sp. NBRI9513R7 were isolated and enumerated from the 45-day-old seedlings, there would still be the question as to where colonization of the root was occurring. Despite the unique advantages that antibiotic resistant marked mutants of wild-type strains offer, such as isolation and enumeration from non-sterile soils, using this technique does not provide adequate information as to where root colonization of the potential PGPR is occurring.

### **Review of Means of Monitoring Endophytic Populations, Case Studies**

Endophytic bacteria are important components of PGPR research. Studies of endophytic bacteria provide methods for monitoring populations inside plants. Summaries of the following studies show some of the various methods used to investigate colonization of plants by endophytic bacteria.

#### ***Endophytic Colonization by *Herbaspirillum frisingense****

##### **Overview of Study**

In a study conducted by Rothballer et al. (2008), endophytic colonization of the diazotrophic betaproteobacterium *Herbaspirillum frisingense* was investigated. To demonstrate endophytic colonization of *H. frisingense*, immunological labeling techniques using monospecific polyclonal antibodies against two *H. frisingense* strains and GFP-tagging were applied. Using the polyclonal antibodies, the *in situ* identification and detailed localization of the two *H. frisingense* isolates within the roots of *Miscanthus x giganteus* seedlings were determined. Three days after inoculation, cells were found inside root cortex cells. At 7 days after inoculation, colonization of the vascular tissue in the central cylinder could be observed. *Gfp*-tagged *H. frisingense* strains could be detected and localized in uncut root material by

confocal laser scanning microscopy. The two strains were found as endophytes in cortex cells, intercellular spaces, and the central cylinder of barley roots. The results of this study demonstrate possible methods that can be used to monitor endophytic colonization. However, the condition in which this study was performed was gnotobiotic. Thus, the potential of *H. frisingense* for endophytic colonization of plants growing in soil was not addressed.

### **Details of Study**

To demonstrate the endophytic colonization of *H. frisingense*, two strains were isolated from either washed roots (Mb group) or from washed leaves and stems (GSF group) belonging to *Miscanthus* spp. growing in Freising, Germany. *Herbaspirillum frisingense* strain GSF30<sup>T</sup> (DSM 13128) was isolated from washed, crushed leaf material and strain Mb11 (DSM 13130) from washed, crushed roots of *M. sacchariflorus* on a nitrogen-free, semi-solid medium. All isolates of *Herbaspirillum* were grown at 30°C on an NB complex medium. Strains that were transposon-tagged were grown with the addition of 50 µl of kanamycin. In addition to the strains of *Herbaspirillum*, two strains of *Escherichia coli* were cultured for plasmid conjugation. *Escherichia coli* strain HB101, with the plasmid pRK600, was grown on Luria-Bertani (LB) medium containing 10 µg mL<sup>-1</sup> chloramphenicol. *Escherichia coli* strain MV1190, harboring the plasmid pJBA28, was cultivated on LB medium supplemented with 50 µg mL<sup>-1</sup> kanamycin.

The plants to be inoculated in this study were *Miscanthus x giganteus* and *M. sinensis* ‘Goliath.’ Plant tissue was obtained by meristem and subsequent callus cultures from shoot tips. Inoculation of these sterile tissue culture plants was performed after 2-3 weeks of growth on MS-medium until fine roots had developed. In addition to *Miscanthus* spp., seeds of barley were also used for monitoring *H. frisingense* endophytic colonization. To eliminate contaminating microorganisms, the barley seeds were disinfested by surface sterilization with 70% ethanol for 5

minutes followed by washing three times with sterile deionized water. Seeds were then incubated in a sodium hypochlorite solution (6-14% Cl active) for 20 minutes and washed five times with sterile deionized water. The seeds were incubated for 3-4 days at 30°C in the dark on NB plates. Only those seeds that showed no visible contamination were chosen for incubation.

*Miscanthus* and barley plants were inoculated when roots of each plant were between 1 and 2 cm in length. The inoculation method involved the use of overnight cultures of the two *H. frisingense* strains that were washed twice with phosphate-buffered saline (1 x PBS, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 130 mM NaCl, pH 7.2) followed by a dilution of 1 x PBS to obtain a concentration of 10<sup>8</sup> cells mL<sup>-1</sup>. Seeds or calli of *Miscanthus* and barley were incubated in this bacterial suspension for 1 hour before being transplanted. To maintain monoxenic cultivation, sealed glass tubes were used as the planting vessels. Each glass tube was filled with 100 g of autoclaved quartz sand and 10 mL of sterile modified Hoagland's solution. Once transferred from the bacterial suspension, the plants were grown under greenhouse conditions from 3 days to 8 weeks, at which time roots were harvested.

Tagging of *H. frisingense* with a *gfp*-containing transposon was performed using the plasmid pJBA28, harboring a mini-Tn5 transposon with a kanamycin resistant cassette, and a constitutively expressed *gfp* gene under control of a P<sub>A1/04/03</sub> promoter. The plasmid was transferred to *H. frisingense* strains GSF30<sup>T</sup> and Mb11 via conjugative transfer by triparental mating. *Herbaspirillum frisingense* transconjugates were selected by plating on minimal medium (MMAB) agar supplemented with 50 µg mL<sup>-1</sup> kanamycin and 6 µg mL<sup>-1</sup> nalidixic acid. Detection of the *gfp*-tagged *H. frisingense* cells was obtained by using confocal laser scanning microscopy (CLSM). To visualize the *gfp*-tagged cells, freshly harvested whole roots or hand-cut root slices were used. Whole roots or slices were embedded in Citifluor, and GFP-derived

fluorescence was detected using a CLSM. GFP was excited at 488 nm by an argon ion laser. Two helium neon lasers provided excitation wavelengths of 543 and 633 nm, which resulted in unspecific autofluorescence of the root material, enabling visualization of the root structure. The three signals were combined and depicted as a red green blue (RGB) image. The results of the GFP-labeling system showed that cells of *H. frisingense* could be found in great numbers on the root surface and in all parts of the inner tissue. Frequently, cells were localized in high numbers in intercellular spaces of the outer cortex but also within intact root cells. In many cases, massive colonization of xylem vessels within the central cylinder was recognized, especially in older parts of the root. Using *gfp*-labeled *H. frisingense* cells, no detectable differences were observed between the colonization patterns of strain GSF30<sup>T</sup> and Mb11.

Immunogold labeling and detection of *H. frisingense* were carried out by first preparing plant material for fixation. Plant material was cut into 1-cm pieces, and the material was washed thoroughly with 1 x PBS and fixed with 10 mL of 1 x PBS containing 3% (w/v) paraformaldehyde and 0.1% (w/v) glutaraldehyde at 4°C overnight. Fixation was stopped by washing with 50 mM NH<sub>4</sub>Cl in 1 x PBS. Samples were dehydrated with rinsing ethanol concentrations (70%, 90%, and 100%, 10 minutes each) and then were embedded in a series of Unicryl dilutions (ethanol: Unicryl 2:1, 1:2, and pure Unicryl, 1 hour each). The plant pieces were then transferred to gelatin capsules and infiltrated with Unicryl overnight. The final step was to allow the samples to solidify using a temperature of 55°C for 2 days. For transmission electron microscopy (TEM), ultra-thin sections (60-80 nm) were prepared with an ultramicrotome and placed on a TEM grid. Subsequently, immunogold labeling was conducted using the previously prepared antisera and an Antirabbit AuroProbe EM with 15-nm gold particles. Contrast staining was performed with uranyl acetate (30 minutes) and lead citrate (4

minutes). Prepared samples were examined with a Zeiss TEM 10CR. For light microscopy, semi-thin sections (0.5-0.7  $\mu\text{m}$ ) were made, heat-fixed on glass slides, and subjected to immunogold labeling as performed for the TEM samples using 5-nm gold particles. This was followed by a silver enhancement and contrasting slices with 0.1% (w/v) toluidine blue solution containing 0.1% (w/v) borax for 3 minutes, dried, and embedded in Entellan<sup>®</sup>.

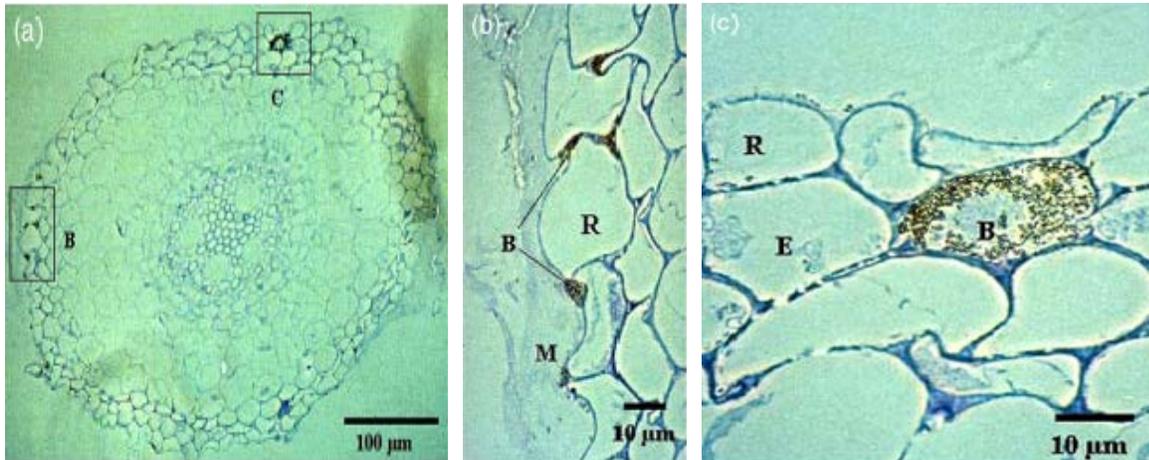


Figure 1. (a) Light microscope image of a root cross-section from a *Miscanthus x giganteus* plant inoculated with *H. frisingense* Mb11, 3 days after inoculation, using immunogold labeling with AntiMb11 and silver enhancement staining. (b) (Detail of a) Bacterial cells (B) of *H. frisingense* Mb11 densely colonized the rhizoplane beneath the mucigel layer (M), penetrated the root surface, and entered the intercellular spaces of the rhizodermis cells (R). (c) (Detail of a) An exodermis cell (B) densely colonized by *H. frisingense* Mb11. Rothballer et al. (2008)

For the endophytic colonization study using immunogold labeling, *Miscanthus x giganteus* plants from axenic tissue cultures were inoculated with *H. frisingense* strains Mb11 and GSF30<sup>T</sup>. Non-inoculated plants and plants inoculated with *Azospirillum brasilense* Sp7 served as negative controls for endophytic colonization. Inoculated plants were grown for 3 days to 8 weeks under greenhouse conditions in monoxenic systems. Examination using light microscopy of immunostained samples after silver enhancement revealed *H. frisingense* cells on the rhizoplane and in outer cortex cells after 3 days and in the endodermis cells and in xylem vessels after 7 days. There was no visible difference in the colonization behavior between the strains Mb11 and GSF30<sup>T</sup>. Electron microscopy and immunogold labeling enabled identification

of the bacteria colonizing root samples as *H. frisingense* strains Mb11 and GSF30<sup>T</sup>. Additionally, TEM enabled documentation of several xylem vessels almost completely filled with bacteria. A comparison of non-colonized xylem vessels showed xylem walls to be intact and not degraded by the presence of the bacteria. In the control plants with *A. brasilense* Sp7, only the rhizoplane was observed to be colonized. When non-inoculated plants were incubated with both polyclonal sera, no unspecific cross-reactions were observed in the plant material. In summary, using methods of immunogold and GFP labeling, Rothballer et al. (2008) were able to determine the colonization pattern of two strains of *H. frisingense*. More specifically, endophytic colonization was detected using both of these techniques. Because the tests were done under monoxenic conditions it is not known if the same techniques could be used to detect colonization in the presence of a native soil microflora.

### ***Endophytic Colonization of Vitis vinifera L. by Burkholderia sp. strain PsJN***

#### **Overview of study**

Another study focused on endophytic colonization of PGPR strains was performed by Compant et al. (2005). The authors investigated the colonization pattern of grape plantlets (*Vitis vinifera* L. cv. Chardonnay) by the plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. Genetically engineered derivatives of strain PsJN tagged with *gfp* (PsJN::*gfp2x*) or *gusA* (PsJN::*gusA11*) genes were used to visualize and enumerate tissue colonization. Epiphytic and endophytic colonization patterns were monitored by dilution plating and microscopic observation of plant organ sections. Utilizing these techniques, the authors first detected bacteria colonizing root surfaces, then inside root tissues, and finally progressing to the fifth internode and fifth leaf tissues. These findings indicate that the wild-type strain PsJN as well as the *gfp*- and *gusA*-marked derivatives endophytically colonized grape plants. However, because the study was

conducted under gnotobiotic conditions, the endophytic colonization potential of strain PsJN in the presence of native microflora remains unknown.

### **Details of study**

To determine the endophytic colonization characteristics of strain PsJN, Compant et al. (2005) first prepared the wild-type strain PsJN for fluorescent and GUS labeling. This was performed by tagging the wild-type PsJN with *gfp* and *gusA* marker genes using mini-Tn5 systems. The wild-type strain PsJN was grown in King's B medium in 5 ml cultures at 20°C until the optical density was 0.7 at 600 nm. The bacterial cells were then pelleted by centrifugation (3,000 x *g*, 10 min, 4°C), washed three times with ice-cold distilled water, and resuspended in 500 µl of ice-cold glycerol. To each 100-µl cell suspension, 200 ng of delivery plasmid DNA was added; the plasmid used was either pUT*gfp*2x, in which two copies of the marker gene were constitutively expressed, or pCAM111, in which *gusA* was under control of the *ptac* promoter. The mixture was then incubated for 15 minutes on ice and subsequently electroporated with a Gene Pulser Plus pulse controller by using settings of 2.5 kV, 200 Ω, and 25 µF. Transformants carrying the GFP marker were selected on King's B medium containing 50 µg of kanamycin per ml (pUT*gfp*2x) or 50 µg of spectinomycin per ml (pCAM111). Colonies and cells of the *gfp*-marked strain were examined with a fluorescence stereomicroscope equipped with a GFP 1 filter and with an optical microscope equipped with a UV light source and a 495 nm fluorescent filter. The *gusA*-marked strain was grown for 4 days at 37°C on King's B medium amended with 50 µg of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt per ml. The bacteria were then examined using an optical stereomicroscope and an optical microscope.

Once the *gfp*- and *gusA*-marked strains of PsJN were created, bacterial growth was determined, and an inoculum for application on grape plantlets was prepared. The *gfp*- and *gusA*-

marked strains of PsJN, as well as the wild-type strain, were grown separately in King's B medium. Each bacterial inoculum was transferred to 100 ml of King's B liquid medium containing the appropriate antibiotic in a 250 ml Erlenmeyer flask and incubated at 20°C on a shaker (150 rpm) for 48 hours. The bacteria were then collected by centrifugation (4,500 x g, 15 min) and washed twice with phosphate-buffered saline (pH 6.5) (PBS). The concentration of each inoculum was then adjusted to approximately  $3 \times 10^8$  CFU/ml with PBS, based on an optical density at 600 nm. The concentration was confirmed by using plate counting.

The plant material selected for this study consisted of disease-free grape plantlets (plantlets free of visible bacterial or fungal contamination) propagated by using nodal explants. These explants were placed in 25-mm-diameter test tubes containing 15 ml of Martin medium. The plantlet cultures were grown in a growth chamber under white fluorescent light ( $200 \mu\text{mol } 52 \text{ m}^2 \cdot \text{s}^{-1}$ ) with a 16-hour photoperiod and a constant temperature of 26°C. Inoculation of the plantlets with PsJN was performed by spreading 200  $\mu\text{l}$  aliquots of bacterial inocula in PBS (wild-type strain PsJN and *gfp*- and *gusA*-marked strains) or PBS (control) on the surface of Martin medium in new test tubes under gnotobiotic conditions. Five-week-old rooted plantlets with five developed leaves were delicately transferred into the test tubes inoculated with bacteria so that only the roots were in contact with the bacterial inoculum. The plantlets were then incubated in the culture chamber as previously described.

To determine endophytic colonization of PsJN, colonization patterns were monitored by dilution plate assays and microscopic observation of plant organs. For dilution plate assays, plantlets inoculated with the *gfp*-marked strain were removed from the agar and were subjected to a surface-sterilization procedure. For surface sterilization, samples were placed in 70% ethanol for 5 minutes (roots) or 3 minutes (fifth internode and fifth leaf), followed by 1% commercial

bleach and a 0.01% Tween 20 solution for 1 minute, and then washed three times in distilled water (1 minute each time). The samples were then ground in sterile Eppendorf microcentrifuge tubes containing 1 ml of PBS for approximately 1 minute followed by shaking for 1 hour (200 rpm) at ambient room temperature. After this period, homogenates were vortexed for 5 seconds, 10-fold serially diluted, and cultured on King's B medium plates supplemented with kanamycin (50 µg/ml). Bacterial colonies were counted after 3 days of incubation at 30°C. Efficacy of the surface-sterilization procedure was determined by culturing the last wash solution from the last rinse. Using dilution plate assays, endophytic colonization of plant organs (roots, fifth internodes, and fifth leaves) were observed at different times. Colonization of the root interior by PsJN::*gfp2x* cells occurred between 1 and 3 hours after inoculation (p.i.) when the population was 6.85 log CFU/g (fresh weight). Endophytic colonization of stems as determined by observing the fifth internode of plantlets showed no detectable PsJN::*gfp2x* cells until 72 hours p.i. At this point, bacteria first appeared and reached the highest population levels around 84 hours p.i. The population of endophytic bacteria present in the fifth internodes of the plantlets was determined to be 5.85 log CFU/g (fresh weight). Endophytic colonization of leaves was not detected until 72 hours p.i. The highest population density occurred 84 hours p.i. at which the population density was determined to be 6.53 log CFU/g (fresh weight).

In addition to dilution plate assays to study endophytic colonization, microscopy was also employed. Preparation of samples for microscopy involved fresh plant organs (roots, fifth internodes, and fifth leaves) of six plantlets inoculated with either the wild-type strain PsJN, the genetic derivatives of this strain (*gfp*- and *gusA*-marked strains), or a control (PBS) collected 96 hours after inoculation. Samples were prepared for microscopic analysis beginning with fixation of the plant organs for 24 hours at room temperature in 2% (wt/vol) glutaraldehyde in 0.1 M

phosphate buffer (pH 7.24) with 2% (wt/vol) sucrose and 0.1% (vol/vol) Tween 20. After this fixation step, samples were rinsed three times (5 minutes each) with phosphate buffer containing 2% (wt/vol) sucrose and then fixed for 4 hours in 1% (wt/vol) osmium tetroxide in phosphate buffer with 2% (wt/vol) sucrose. Samples were then dehydrated in an alcohol series, transferred to acetone, and embedded in araldite. Semithin sections (thickness, 1  $\mu\text{m}$ ) for different treatments were cut using a microtome, collected on glass slides, stained with 0.1% toluidine blue, and examined with a microscope.

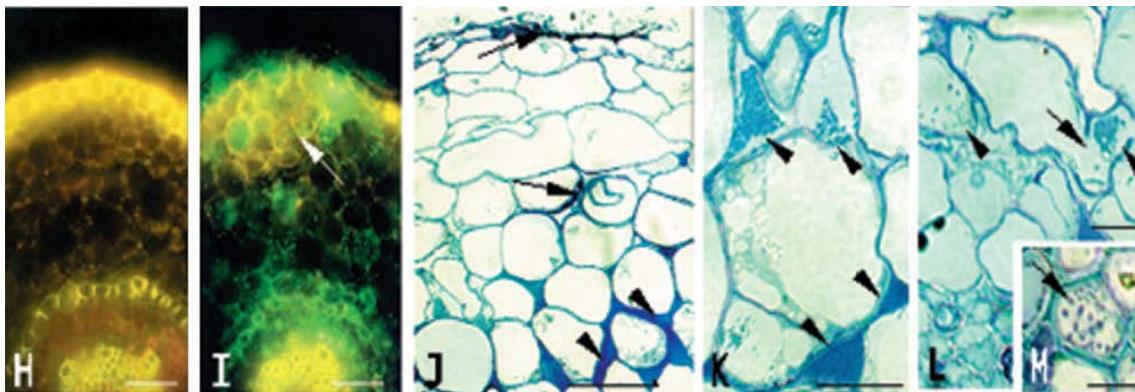


Figure 2. Microphotographs of rhizoplane and endorhiza of grape plantlets after inoculation with strain PsJN. (H and I) Epifluorescence microscope image of primary internal root tissues after treatment with PBS (H) or inoculation with PsJN::*gfp2x* (I). (J to M) Light microscope images of resin-embedded primary roots after inoculation with PsJN, showing colonization in the exodermis and cell wall of a cortical cell (J), intercellular colonization of cortical cells (J and K) (arrowheads), a break in the endodermis (arrow) caused by PsJN (arrowheads), and PsJN in xylem vessels (M) (arrow). (H and I) Bars = 100  $\mu\text{m}$ ; (J,K, and L) bars = 50  $\mu\text{m}$ ; (M) bar = 20  $\mu\text{m}$ . Compant et al. (2005)

Upon examination, a yellow autofluorescence was observed at 96 hours p.i. in the rhizodermis, endodermis, and xylem vessels of primary roots in control plantlets as a result of phenolic compounds that fluoresced under UV light. Yellow autofluorescence was also observed in the primary roots of plantlets inoculated with PsJN::*gfp2x*. However, several cortical cell layers exhibited additional yellow fluorescence in plantlets inoculated with PsJN::*gfp2x* compared to the nonbacterized control treatment. Due to large bacterial populations that developed on the rhizoplane, bacterial colonization of internal root tissues could not be definitively confirmed microscopically by using hand-cut sections of plantlets inoculated with

PsJN::*gfp2x* because cells might have been introduced from the external root surface during sample preparation. Despite this, microscopic analyses of resin-embedded roots demonstrated that bacterial cells followed an inter- and/or intracellular colonization pattern in cortical cells, endodermis, and xylem vessels in contrast to control samples where bacteria were not present. Microscopic observations of the fifth internode of stems revealed a green autofluorescence (epidermis), red autofluorescence (parenchyma), and yellow autofluorescence (vascular system) in both nonbacterized plantlets and in PsJN::*gfp2x*-bacterized plantlets. Observations at higher magnifications from the epidermis to xylem vessels revealed that PsJN::*gfp2x* or wild-type PsJN cells were found only in xylem vessels, in contrast to nonbacterized samples, in which no bacteria were observed. Additionally, no blue color was observed in the fifth internode of nonbacterized plantlets after incubation with the GUS substrate. A blue color was observed 96 hours p.i. in plantlets inoculated with the PsJN::*gusA11* strain. When observing endophytic colonization of the fifth leaves of plantlets, the same autofluorescence observed in the fifth internode occurred in both the control treatment and treatments with PsJN::*gfp2x* or wild-type PsJN cells. Bacterial cells were detected in xylem vessels in the PsJN::*gfp2x* and wild-type PsJN treated plantlets in contrast to nonbacterized samples. Endophytic colonization of the fifth leaf of plantlets was also observed using the PsJN::*gusA11* construct in which a blue color could be observed 96 hours p.i. in primary and secondary veins. This is in contrast to nonbacterized plantlets that exhibited no blue color after incubation with the GUS substrate.

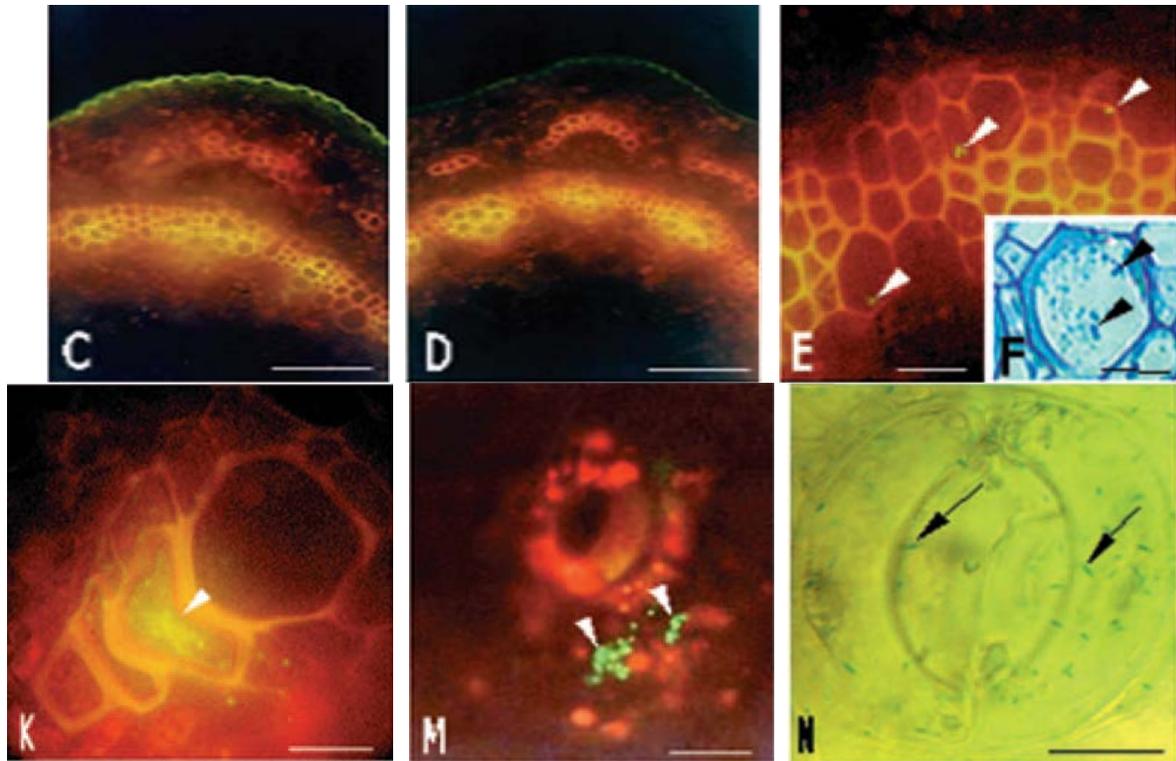


Figure 3. Images of the fifth internode and leaf internal tissues of grape plantlets after rhizosphere inoculation with PsJN. (C to E) Epifluorescence microscope images of hand-cut sections of the fifth internode after treatment with PBS (C) or after inoculation with PsJN::*gfp2x* (D and E), showing PsJN::*gfp2x* cells in xylem vessels (E) (arrowheads). (F) Light microscope image of resin-embedded stem after inoculation with wild-type strain PsJN, showing cells in xylem vessels (arrowheads). (K) Epifluorescent microscope image of hand-cut section of the fifth leaf after inoculation with PsJN::*gfp2x* showing cells in xylem vessels (arrowhead). (M) Epifluorescence microscope image of fifth leaf after inoculation with PsJN::*gfp2x* (M) showing bacteria exiting from stomata (arrowheads). (N) Light microscope image of fifth leaf after inoculation with PsJN::*gusA11* showing blue cells (arrows) under stomata. (C and D) Bars = 150  $\mu\text{m}$ ; (E and F) bars = 30  $\mu\text{m}$ ; (K) bar = 20  $\mu\text{m}$ ; (M) bar = 10  $\mu\text{m}$ ; (N) bar = 5  $\mu\text{m}$ . Compant et al. (2005)

Endophytic colonization of grape plantlets by the plant growth-promoting bacterium *Burkholderia* sp. strain PsJN was examined using *gfp*- and *gusA*-tagging. The rhizospheres of 4- to 5-week-old plantlets with five developed leaves were inoculated with bacterial suspensions. Epiphytic and endophytic colonization patterns were then monitored by dilution plating assays and microscopic observation of plant organ sections. Using these techniques, a pattern of bacterial colonization could be determined. Bacteria were first detected on root surfaces, then in internal root tissues, and finally in the fifth internode and tissues of the fifth leaf. Bacteria were not observed on the surfaces of stems or leaves but could be detected in xylem vessels of the fifth

internode and fifth leaf of plantlets. In summary, the use of GFP and GUSA labeling enabled Compant et al. (2005) to determine the colonization pattern of *Burkholderia* strain PsJN. The methods used followed epiphytic and endophytic patterns of colonization in *Vitis vinifera* L. plantlets. However, the condition in which this study was conducted was gnotobiotic in nature. Thus, it is not known if the same techniques could be applied to detect colonization in the presence of native soil microflora.

### Summary

A number of techniques can be employed to study bacterial colonization patterns in plant studies. The systems reviewed here include marking systems such as GFP, GUS, and FISH for visualizing bacteria on plants. In addition, antibiotic resistant-marked systems allow culturing the inoculated bacterial strain present in a background of native soil microbes.

Each system of studying bacterial colonization has advantages and limitations. In order for a system utilizing GFP to work, bacterial strains being studied must be successfully tagged by a conjugative plasmid bearing a fluorescence gene. For example, in the study conducted by Timmusk et al. (2005) two *P. polymyxa* isolates, B1 and B2, were initially tagged with two plasmids. While both plasmids were able to be established in both isolates of *P. polymyxa*, isolate B2 failed to exhibit measurable levels of fluorescence due to inefficient expression of the *gfp* gene. In addition, some bacterial strains of interest cannot be transformed with conjugative plasmids. Hence, establishing a GFP system that can be useful for studying colonization is not straightforward.

Other systems of monitoring colonization pose equally challenging issues. Systems using FISH require the use of oligonucleotide probes that target specific regions of a genomic sequence. Some probes, such as PSE-225 used by Buddrus-Schiemann et al. (2010), target a

range of sequences from several different species of bacteria. This is to say that this probe targets not only *Pseudomonas* sp. DSMZ 13134 but numerous other *Pseudomonas* species as well as bacteria belonging to different genera. The study used monoxenic conditions in order to study the colonization pattern of strain DSMZ 13134 so as to avoid possible probe hybridization to microorganisms containing a closely related sequence. Antibiotic resistant-marking is also one of many methods used to monitor bacterial colonization. Antibiotic resistance offers the advantage of recovery and enumeration of bacteria in ecological studies so long as the resistant strain is equal to the wild-type strain in regards to organism fitness, something that is nearly impossible to confirm. One of the disadvantages of this system is that bacteria cannot be visualized microscopically as can be performed using a system such as GFP or FISH. Considering this, the exact location of bacterial populations within the rhizosphere can be difficult to establish using antibiotic resistance alone. As this review of the literature indicates, each system of monitoring bacterial colonization has its own unique advantages and disadvantages.

### III. MATERIALS AND METHODS

Greenhouse and microplot experiments were conducted to evaluate the root colonizing characteristics of *Bacillus firmus* strain GB126. Detection of GB126 on roots growing in nonsterilized soils was based on using a spontaneous rifampicin-resistant mutant of the strain. Visualization of colonization patterns was done by pressing roots onto agar with rifampicin, and quantification was done by making dilution platings of roots onto agar with fungicides and rifampicin.

**Bacterial strains.** *B. firmus* strain GB126 was provided by Bayer CropScience, Research Triangle Park, NC. Spontaneous rifampicin-resistant mutants of GB126 were generated by growing bacteria in 50 ml of tryptic soy broth (TSB) (Bectin, Dickinson and Company, Sparks, MD) using 250 ml Erlenmeyer flasks. Bacteria were grown with shaking in ten different flasks for 24 h at which point rifampicin was added to each flask in concentrations of either 100 or 200 µg/ml. Flasks were incubated with shaking for another 2-5 days until turbidity was evident. From each flask, a loopful of inoculum was streaked onto tryptic soy agar (TSA) plates amended with 100 µg/ml rifampicin (TSArif). The TSArif plates were incubated at 28 C for 48 h, and colonies with similar growth rates and morphology as the wild-type GB126 were selected. Each of the selected colonies was again streaked onto TSArif, and the wild-type strain was streaked onto TSA at the same time. Two rifampicin-resistant mutant strains (GB126 R1 and R2) were selected based on their similarities to the wild-type strain, and these mutants were used in the

experiments described below. Typical colony morphology of GB126 rif mutants was described at 48 h on TSArif, pH 8.0. The morphology of the colonies on the plates were as follows: 2-3 mm in size, flat, slightly glistening, non-pigmented with darker centers, round with margins not quite entire, somewhat serrate, colonies exhibit a slightly grainy appearance (Figure 4). GB126 R1 and R2 were maintained at -80°C in TSB amended with 20% glycerol. Cultures for each of the experiments were obtained by plating from the frozen stocks.

**Inoculum Preparation.** To prepare inoculum for seed application, GB126 R1 and R2 were taken from -80°C storage and streaked onto TSA. The plates were incubated at 28.0°C for 24 h. Following this incubation period, a loopful was transferred from each plate to a 125 ml Erlenmeyer flask containing 50 ml of TSB + 100 µg/ml rifampicin with a pH of 8.0. Flasks inoculated with GB126 R1 and R2 were placed on a shaker (120 rpm) for 48 h when turbidity was observed. Seeds were inoculated with 1.0 ml of this culture.

**Plant material.** Root colonization by GB126 R1 and R2 was assessed on corn ('Axis 59J25'), cotton ('Phytogen 565 WRF'), and soybean ('Asgrow 6702 RR').

**Planting medium.** Seeds were planted in a 1:1 ratio of field soil (clay loam) to Sunshine #2 Natural & Organic (Sun Gro Horticulture, Canada) in varying pot sizes. For sampling at 1 and 2 WAP, 4-inch square pots were used, while 6-inch round pots were used for sampling at 4 WAP. Two seeds per pot were planted to ensure adequate germination.

## **Assessing Sporulation of Rifampicin-Resistant GB126 Mutants**

As part of evaluating the rifampicin-resistant mutant strains of GB126, the two mutants used in this study were tested for sporulation capacity. The two rif-resistant strains of GB126, R1 and R2, were grown using the methods described in creating the bacterial strains for seed inoculation (above). Six 250 ml Erlenmeyer flasks containing 50 ml of 50% TSB (pH 8.0) containing 100 µg/ml rifampicin were used to create broth cultures for this test. The two rif-resistant GB126 strains R1, R2, or a combination of the two were inoculated into the flasks and were incubated at room temperature while shaking at 120 rpm.

To determine sporulation of the GB126 mutant strains, pasteurization was performed to eliminate vegetative cells prior to dilution plating. Serial dilutions were prepared by taking 1 ml of broth from the three cultures (R1, R2, or the R1/R2 combination). The dilutions were divided into two treatments: a non-pasteurized and pasteurized treatment. Pasteurization was performed by placing the test tubes containing the dilutions of the three cultures in a hot water bath (80°C) for 20 min. Once pasteurization was complete, the cultures from both treatments were plated onto 50% TSA and incubated at 28°C for 48 h, and all isolated colonies were then enumerated. This process was performed at 3, 7, 10, and 14 days after inoculation (DAI).

## **Optimization of Root Printing**

Experiments were designed to evaluate and optimize “root printing” as a means of visualizing colonization of root sections with GB 126 rif. Root printing is the term used for the process of placing onto agar sections of roots from plants inoculated with GB126 rif and allowing the strain to grow. Root printing was done on corn, cotton, and soybean in greenhouse conditions at the Plant Science Research Center (PSRC) of Auburn University. Printing was

performed by extracting plant roots at various sampling times. Roots of the tested crops were washed and cut into sections approximately 1-2 cm in length. Sections of the root were placed onto an appropriate isolation agar (described below) for GB126 rif. Potential growth of GB126 rif colonies was observed using photographic evidence.

Three experiments were conducted. Experiment one tested root printing on corn, cotton, and soybean at 1 WAP onto 50% TSA with and without rifampicin. Experiment two tested root printing on corn, cotton, and soybean at 2 WAP utilizing 50% TSArif with and without fungicides present. Experiment three tested root printing on corn, cotton, and soybean at 4 WAP using 50% TSArif with different fungicides.

Experiment one tested “root printing” of corn, cotton, and soybean using two media. One week after planting, plant roots were extracted from pots by manually removing the soil by hand. Excess soil was removed by shaking the roots, leaving some soil attached. Following root extraction, sections of the root system from each crop were “printed” onto 50% TSA and 50% TSArif plates. “Printing” involved carefully laying each root section onto the agar surface so that all parts of the root were in contact with the agar. The plates were then incubated for 48 h at 28.0°C when the presence of absence of colonies with the typical morphology of GB126 rif were noted.

Experiment two test root printing again with the inclusion of fungicides to the isolation media. Similarly to the first experiment, plant roots were manually extracted from pots, and excess soil was removed by hand and gentle shaking. The roots of each crop were then divided into 1-2 cm sections, and two sections were plated onto 50% TSArif and 50% TSArif with fungicides The fungicides used included metalaxyl at 25 µg/ml and ipconazole at 25 µg/ml.

After root sections were printed, plates were incubated for 48 h at 28.0°C before observations were made. Evidence of growth of GB126 rif was documented using photography.

Experiment three tested root printing using a medium with different fungicides than those used in experiment two. The same sampling procedure for extracting plant roots was performed as in the first two experiments. Five root sections were plated per plate. For this experiment, the fungicide ipconazole was omitted and thiram was introduced. Root sections were printed onto 50% TSArif with metalaxyl at 100 µg/ml, thiram at 25 µg/ml, or a combination of both. After incubation for 48 h at 28.0°C, plates were examined for the presence of GB126 rif. Growth of GB126 rif on the root-printed agar plates was documented photographically.

### **Dilution Plating for Quantifying External and Internal Root Colonization**

Four experiments were designed to quantify populations of GB126 rif, using log colony forming units (CFU) per gram of root or soil. Experiments were conducted with corn, cotton, and soybean as described above. Dilution plating was performed by extracting plant roots at various times as described below and plating onto 50% TSA, pH 8.0 amended with rifampicin at 100 µg/ml, metalaxyl at 100 µg/ml, and thiram at 25 µg/ml (unless otherwise noted below).

In each test, seeds were planted in a 1:1 ratio of field soil (clay loam) to Sunshine #2 Natural & Organic (Sun Gro Horticulture, Canada) potting mix in varying pot sizes. For sampling at 6 WAP, #1000 series (Nursery Supplies, Kissimmee, FL) pots were used and #2000 series pots for 8, 10, and 12 WAP. Two seeds per pot were planted to ensure adequate germination.

Experiment one quantified populations on the rhizoplane and the rhizosphere of corn, cotton, and soybean at 6 WAP. For this experiment dilution plating of corn, cotton, and soybean

was performed for the first time to see if GB126 colonization could be enumerated. The roots of the three crops were subjected to two different sampling methods. The sampling methods involved were designated as “rhizoplane” or “rhizosphere” depending on the method of extraction for dilution plating. The “rhizoplane” sampling method involved extracting the roots from the pots and washing them using tap water from a hose at the PSRC to remove adhering soil. The “rhizosphere” sampling method involved extracting the roots from pots and gently shaking them, leaving behind attached soil particles. Samples were then cut into sections and agitated for approximately 20 s in 9 ml of sterile water in a test tube, then subjected to serial dilutions followed by plating onto the GB126 rif isolation medium using aliquots of 50  $\mu$ l per dilution. Plates were placed in an incubator at 28.0°C for 48 h before colonies were counted. The populations of GB126 rif were calculated using the formula  $\log(\text{CFU/g} + 1)$ .

Experiment two, on soybean, quantified populations, at 8 WAP, from the rhizosphere, rhizoplane (root surface), rhizosphere and endorhiza (inside the root) and endorhiza. The “rhizosphere” and the “rhizoplane” samples were taken as described above. The “rhizosphere and endorhiza” sampling involved the same extracting roots from pots and removing excess soil by shaking and then triturated (no surface disinfestation) using a Kleco® grinder (model 4200, Garcia Manufacturing, Visalia, CA) for 1-2 min. The “endorhiza” sampling method involved washing roots using tap water followed by a surface disinfestation treatment. For disinfestation, roots were subjected to a 96% ethanol solution for 30 s, a 20% bleach solution for 60 s, and then a series of five distilled water rinses. The roots were then triturated using a Kleco® grinder for 1-2 min. The crop chosen for this experiment was soybean only. Six replications were performed for each sampling method. Plates were allowed to incubate for 48 h at which point colony counts were enumerated. Bacterial population sizes were compared using a one-way analysis of

variance (ANOVA) at  $P = 0.01$  and  $P = 0.05$ . Statistical comparisons were made using JMP® software (SAS Institute Inc., Cary, NC, USA, 2012).

Experiment three quantified populations on the rhizoplane and endorhiza of corn and cotton at 10 WAP. This experiment involved using dilution plating using two different sampling methods to enumerate GB126 rif colonies. The two methods used were sampling “rhizoplane” and “endorhiza” populations. For sampling the “rhizoplane,” roots were washed using tap water. For sampling “endorhiza,” roots were washed using tap water, subjected to surface disinfection, and triturated using a Kleco® grinder. The roots of corn and cotton were sampled with four replicates used for each sampling method. Plates were allowed to incubate for 48 h at which point colony counts were enumerated. Bacterial population sizes were compared using a Student’s t-test at  $P = 0.01$  and  $P = 0.05$ . Statistical comparisons were made using JMP® software.

Experiment four quantified populations on the rhizoplane and endorhiza of corn, cotton, and soybean at 12 WAP. For this experiment, dilution plating was utilized involving the same sampling methods as used in experiment 3. Corn, cotton, and soybean were sampled with four replicates used per sampling method. Plates were allowed to incubate for 48 h at which point colony counts were enumerated. Bacterial population sizes were compared using a Student’s t-test at  $P = 0.01$  and  $P = 0.05$ . Statistical comparisons were made using JMP® software.

### **Comparative Colonization of Soybean in Acidic Soils**

An experiment was designed to test the colonization of GB126 rif in soils with different pH values. Specifically, this experiment was designed to determine colonization capacity in acidic soils. To determine colonization in soils with different pH values, root printing was

performed with modifications from previous experiments conducted using this technique. In contrast to previous root printing experiments, whole root systems would be printed as opposed to excised sections of the roots. In addition, the agar concentration was modified to allow the root systems to be placed beneath the surface of the agar. To study colonization on whole root systems, soybean was chosen due to the growth of a central taproot and hardness. Soybean seedlings were grown in greenhouse conditions at the PSRC and harvested 14 days after planting (DAP) for printing.

**Planting medium.** Soybean seeds were planted in five different soil types from different field stations. The soil types used were: a 1:1 ratio of field soil (clay loam) to Sunshine #2 mix (pH 7.05) (control soil); Blackbelt (31.2-40.4-28.4%, pH 6.9); Piedmont (52-25-23%, pH 5.15); Sand Mountain (56-12-33%, pH 5.05); Wiregrass (57-15-28%, pH 6.15). Seeds were sown in 20 oz (590 ml) polystyrene cups (Dart Container Company, Mason, MI) with holes punched in the bottom for drainage.

**Isolation medium.** A new formulation of fungicides was used for testing during this test. The improved isolation medium used consisted of 50% TSB, pH 8.0, with rifampicin at 100  $\mu\text{g/ml}$  and the agar concentration adjusted to 24% to enable root system printing beneath the agar surface. Additionally, the medium was amended with metalaxyl at 100  $\mu\text{g/ml}$ , pentachloronitrobenzene (PCNB) at 100  $\mu\text{g/ml}$ , and pimaricin at 10  $\mu\text{g/ml}$ .

In the single experiment to test the colonization capacity of GB126 rif in soils with different pH values, soybean seeds were planted in five different soils with pH values ranging from 5.05 to 7.05. Plants were growth for 14 days at which point the soybeans were extracted from the planting containers by using a knife to cut the cups in half around the soil. The two cup halves were removed and the soil was gently removed by hand. The shoot of each plant was then

separated from the root system by cutting directly above the crown. The root system was then placed onto a 100 mm<sup>2</sup> plate containing the isolation medium described above. The roots were then pressed beneath the agar surface using a sterile inoculating needle. Plates were incubated at 28.0°C for 48 h. Colonization of GB126 rif on the root systems of the soybean plants was documented using photography.

### **Microplot Trials to Confirm Colonization Capacity**

Two trials were conducted using microplot trials to confirm the colonization capacity of GB126 rif as seen in previous greenhouse trials at Auburn University's PSRC. The first trial was designed to determine colonization in corn, cotton, and soybean using a single soil type. The second trial was designed to determine colonization in soybean in five different soil types with varying soil textures and pH values. The purpose of microplot trials was to determine colonization in conditions that would be more closely related to those experienced in field trials. For both trials, roots were sampled at 3, 6, and 12 WAP to determine patterns of colonization of GB126 rif.

**Planting medium for microplot trial 1.** Corn, cotton, and soybean seeds were planted in a 3:1 ratio of field soil from E.V. Smith (sand-clay-silt: 86.8-2.4-10.8%) to sand (pH 5.8). Pot sizes used were #2000 series (Nursery Supplies, Kissimmee, FL) for planting nested inside #1600S series pots situated in mulch. Two seeds per pot were planted to ensure adequate germination.

**Planting medium for microplot trial 2.** Soybean seeds were planted in five different soil types from different field stations. The soil types used were: a 3:1 ratio of field soil from E.V. Smith (sand-clay-silt: 86.8-2.4-10.8%) to sand (pH 5.8) (control soil); Blackbelt (31.2-40.4-

28.4%, pH 6.9); Piedmont (52-25-23%, pH 5.15); Sand Mountain (56-12-33%, pH 5.05); Wiregrass (57-15-28%, pH 6.15). Pot sizes used were #2000 series (Nursery Supplies, Kissimmee, FL) for planting nested inside #1600S series pots situated in mulch. Two seeds per pot were planted to ensure adequate germination.

**Isolation medium.** The standardized medium used for isolating GB126 rif for dilution plating was 50% TSA, pH 8.0 amended with rifampicin at 100  $\mu\text{g/ml}$ , metalaxyl at 100  $\mu\text{g/ml}$ , pentachloronitrobenzene (PCNB) at 100  $\mu\text{g/ml}$ , and pimarinic at 10  $\mu\text{g/ml}$ .

Microplot trial 1 quantified populations on the rhizoplane and endorhiza of corn, cotton, and soybean at 3, 6, and 12 WAP. For this experiment, dilution plating was performed as in the previous greenhouse experiments. Corn, cotton, and soybean were sampled with six replicates used per sampling method. Plates were allowed to incubate for 48 h at which point colony counts were enumerated. Bacterial population sizes were compared using the mixed models procedure of PC-SAS (SAS Institute Inc., Cary, NC, USA, 2012).

Microplot trial 2 quantified populations on the rhizoplane and endorhiza of soybean at 3, 6, and 12 WAP in five different soil types. For this experiment, dilution plating was performed as in the previous greenhouse experiments. Soybean was sampled with five replicates used per sampling method. Plates were allowed to incubate for 48 h at which point colony counts were enumerated. Bacterial population sizes were compared using the mixed models procedure of PC-SAS.

## IV. RESULTS

### **Assessing Sporulation of Rifampicin-Resistant GB126 Mutants**

The results of this test showed variation of the GB126 rif strains regarding sporulation. During the first assessment of bacterial populations at 3 DAI, colonies grew from non-pasteurized samples but not from those that were pasteurized. At 7 DAI, colonies were observed from both the pasteurized and non-pasteurized treatments. The 7 DAI populations per ml of non-pasteurized cultures were log 7.54 for R1, log 7.57 for R2, and log 7.73 for the combination. For the pasteurized cultures, populations per ml were log 4.42 for R1, log 4.27 for R2, and log 4.64 for the combination. The 10 DAI populations per ml of non-pasteurized cultures were log 7.78 for R1, log 7.75 for R2, and log 7.62 for the combination. The corresponding populations per ml for the pasteurized cultures were log 4.85 for R1, log 4.94 for R2, and log 4.66 for the combination. During the final sampling period at 14 DAI, the populations per ml of the non-pasteurized cultures were log 7.03 for R1, log 7.76 for R2, and log 5.28 for the combination. The populations per ml for the pasteurized cultures were log 4.30 for R1, log 4.77 for R2, and log 3.15 for the combination. From this data, the estimated spore counts of the rif mutants were typically 3 log units lower than the vegetative cell counts.

### **Optimization of Root Printing**

**Experiment 1.** In this initial test aimed at determining if pieces of roots could be placed directly on agar to observe growth of GB126 rif on roots, of the presence of GB126 rif was not

observed after 48 h incubation at 28.0°C. On both the TSA plates, numerous bacterial colonies with different morphologies were present, so the presence of the inoculated strain could not be differentiated from background soil bacteria. In addition, presence of fast-growing fungal colonies, beginning at 48 hr after plating, made it impossible to observe colony morphologies of the suspected GB126 rif on TSArif plated. From this test, it was determined that further testing would focus mainly on the use of TSArif plus fungicides.

**Experiment 2.** In this experiment, unlike in experiment one, some colonies of GB126 rif could be observed growing on root sections of cotton and soybean on agar plates containing 50% TSArif without fungicides (Figure 5). In contrast, with corn root sections prolific and rapid fungal growth prevented observations of any potential growth of GB126 rif colonies on agar without fungicides. When root sections were placed on 50% TSArif with metalaxyl and ipconazole, both at 25 µg/ml, no growth of GB126 rif colonies was observable on root sections from any of the three crops. It was determined that while fungicides are necessary enable observation of GB126 rif growth after incubation for several days, the choice of fungicides needed to be changed.

**Experiment 3.** In this test, where the fungicides used in TSA-rif were changed to metalaxyl at 100 µg/ml, thiram at 25 µg/ml, or both, colonies of GB126 rif could clearly be observed growing on roots placed on agar plates after 48 h incubation at 28°C. The new fungicides used did not interfere with the growth of GB126 rif unlike previous fungicides used. Colonies of GB126 rif could be seen on corn, cotton, and soybean (Figure 6) treated with GB126 rif whereas no colonies were detected on root pieces from untreated controls of each crop. It was concluded that thiram and metalaxyl were necessary and sufficient for depressing growth of soil

fungi long enough to allow growth and visualization of GB126 rif on root pieces of all three crops.

### **Dilution Plating for Quantifying Internal and External Root Colonization**

**Experiment 1.** Population sizes of GB126 rif were calculated in the rhizosphere and on the rhizoplane of corn, cotton, and soybean at 6 WAP. Dilution plating on 50% TSA + rifampicin (100 µg/ml), metalaxyl (100 µg/ml), and thiram (25 µg/ml) allowed growth and quantification of GB126 rif. The calculated log CFU/g root of GB126 rif in the rhizosphere was 5.31 for corn, 6.81 for cotton, and 5.71 for soybean (Table 1). Population densities of GB126 on the rhizoplane were 5.58 for corn, 6.25 for cotton, and 6.08 for soybean (Table 1).

**Experiment 2.** In this test with soybean, population densities at 8 WAP were calculated for the rhizosphere, rhizoplane (root surface), rhizosphere and endorhiza (inside the root), and the endorhiza only (surface disinfestation). Populations of GB126 rif ranged from log 7.17 on the rhizoplane to 4.75 in the endorhiza (Table 2), and this difference was significant ( $P = 0.01$ ). Interestingly, populations of GB126 rif in the rhizosphere were not significantly greater than populations on the rhizoplane.

**Experiment 3.** Population densities of GB126 rif on the rhizoplane and in the rhizosphere of corn and cotton were evaluated at 10 WAP. On corn, population densities were significantly higher on the rhizoplane than in the endorhiza at  $P = 0.05$ , while on cotton, there the rhizoplane and rhizosphere populations were not significantly different (Table 4)

**Experiment 4.** At 12 WAP population densities of GB126 rif were assessed on the rhizoplane and in the endorhiza on corn, cotton, and soybean. On corn, the population density on the rhizoplane was significantly higher ( $P = 0.01$ ) than in the endorhiza (Table 5). With both

cotton and soybean, there were no significant differences between populations on the rhizoplane and in the endorhiza at  $P = 0.05$  (Tables 6 and 7). The test demonstrated that GB126 rif colonized the rhizoplane and the endorhiza of the three test crops for the 12 week period of the experiment.

### **Comparative Colonization of Soybean in Acidic Soils**

The use of 100 mm<sup>2</sup> semi-solid agar plates allowed visualization of root colonization by GB126 rif on whole root systems of soybean seedlings (Figures 7 – 9). After incubation for 48 hr, colonies of GB126 rif colonies were seen growing on and near the root systems of soybean that had been planted in five different soil types, each possessing a different pH value. The soils used for this experiment consisted of the following: a 1:1 ratio of field soil (clay loam) to Sunshine #2 mix (pH 7.05) (control soil); Blackbelt (31.2-40.4-28.4%, pH 6.9); Piedmont (52-25-23%, pH 5.15); Sand Mountain (56-12-33%, pH 5.05); Wiregrass (57-15-28%, pH 6.15). The degree to which GB126 rif grew from the soybean root systems varied. Major GB126 rif colonization was observed on the agar plates imprinted with soybean roots from the control (Figure 7, upper images) and Sand Mountain (Figure 8, lower images) soil types while growth was barely visible on plates containing roots from the Piedmont (Figure 8, upper images) soil type. However, despite the degree of colonization, this test confirmed that GB126 rif was capable of colonizing soybean roots in soils with low pH values.

### **Microplot Trials to Confirm Colonization Capacity**

**Microplot Trial 1.** In this trial, the population sizes of GB126 rif on the rhizoplane and in the endorhiza were assessed with corn, cotton, and soybean at 3, 6, and 12 WAP in one soil

type. With corn, varying population densities among the rhizoplane and endorhiza at the three sampling times (Figure 10). At 3 WAP, the mean log CFU/g of GB126 rif was 4.56 on the rhizoplane and 3.57 in the endorhiza. At 6 WAP, populations were log 3.82 on the rhizoplane and log 2.51 in the endorhiza. At 12 WAP, the final sampling period, the population densities were log 3.20 on the rhizoplane and log 2.99 in the endorhiza.

With cotton, at 3 WAP, the mean population of GB126 rif was log 3.78 on the rhizoplane and log 0.95 in the endorhiza (Figure 11). At 6 WAP, the population was log 3.56 on the rhizoplane and log 1.67 in the endorhiza. At 12 WAP the population densities were log 3.00 on the rhizoplane and log 1.43 in the endorhiza.

With soybean at 3 WAP, the average population of GB126 rif was log 4.39 on the rhizoplane and log 2.86 in the endorhiza (Figure 12). At 6 WAP, the population was log 4.39 on the rhizoplane and log 1.52 in the endorhiza. At 12 WAP the populations were log 4.08 and on the rhizoplane and log 1.52 in the endorhiza.

**Microplot Trial 2.** The average population sizes of GB126 rif were determined using the formula  $\log(\text{CFU/g} + 1)$  for soybean at three sampling times in five soil types selected to represent a range of pH and textures: a 3:1 ratio of field soil from E.V. Smith (sand-clay-silt: 86.8-2.4-10.8%) to sand (pH 5.8) (“control soil”); Blackbelt (31.2-40.4-28.4%, pH 6.9); Piedmont (52-25-23%, pH 5.15); Sand Mountain (56-12-33%, pH 5.05); Wiregrass (57-15-28%, pH 6.15). Roots were sampled at 3, 6, and 12 WAP to calculate the mean log CFU/g populations of GB126 rif in the rhizoplane and endorhiza.

For the “control soil”, at 3 WAP, average populations of GB126 rif were log 4.45 on the rhizoplane and log 4.15 in the endorhiza (Figure 13). At 6 WAP the populations were log 4.24

on the rhizoplane and log 1.71 in the endorhiza. At 12 WAP, populations were log 3.79 on the rhizoplane and log 2.64 in the endorhiza.

For the Blackbelt soil at 3 WAP, the populations of GB126 rif were log 4.43 on the rhizoplane and log 1.19 in the endorhiza (Figure 14). At 6 WAP, the mean populations were log 3.99 in the rhizoplane and log 2.23 in the endorhiza. Populations at 12 WAP were log 3.96 on the rhizoplane and log 1.10 in the endorhiza.

Colonization of GB126 rif on soybean planted in the Piedmont soil was markedly different than colonization in the other tested soil types. Populations were not detected from the endorhiza at any of the sample times. Additionally, GB126 rif was not detected in the rhizoplane at 3 WAP, and its population in the rhizoplane was log 1.77 at 6 WAP and log 0.58 at 12 WAP (Figure 15).

With the Sand Mountain soil at 3 WAP, the average population of GB126 rif was log 3.71 on the rhizoplane and log 2.09 in the endorhiza (Figure 16). At 6 WAP, populations were log 3.87 on the rhizoplane and log 1.01 for the endorhiza. At 12 WAP populations were log 2.18 on the rhizoplane and log 1.77 in the endorhiza.

With the Wiregrass soil at 3 WAP, population densities of GB126 rif were log 3.81 on the rhizoplane and log 2.77 in the endorhiza (Figure 17). At 6 WAP, populations were log 4.16 on the rhizoplane and log 2.19 in the endorhiza. At 12 WAP population densities were 2.60 on the rhizoplane and log 0.50 in the endorhiza.

## V. DISCUSSION

### Assessing Sporulation of Rifampicin-Resistant GB126 Mutants

The results from testing the sporulation capacity of the GB126 rif mutants suggests that sporulation may have been affected by selecting for a rif-resistant phenotype. Although the degree of sporulation of the GB126 rif strains appeared to be affected, a positive control of the wild-type GB126 strain was not included in this experiment, but from other experiments it would be expected that the wild-type GB126 strain would yield greater than  $10^7$  CFU/g of spores under similar conditions (data not shown). Further evidence of a diminished sporulation capacity of the GB126 rif mutants is demonstrated by comparing sporulation capacity as typically seen in other *Bacillus* sp. For instance, *B. subtilis* has been reported to obtain spore counts of  $10^7 - 10^8$  (Vidwans et al. 1995).

Resistance to rifampicin has been reported to arise from mutations in the nucleotide sequence within the *rpoB* gene encoding the  $\beta$ -subunit of RNA polymerase (Moeller et al. 2012). Mutations of this gene have been shown to lead to alterations in expression in a number of global phenotypes associated with transcriptional control. It is possible that regulation of sporulation could be affected by mutations in the *rpoB* gene, the same gene that may be responsible for conferring rifampicin resistance. Although the mutations associated with the *rpoB* gene appear to effect sporulation in a negative manner with GB126 rif strains, it is uncertain if this is detrimental to the ability of these strains to colonize and persist on plant roots as compared to the wild type strain.

While this test appears to indicate that GB126 rif strains have a diminished capability in sporulation, there could be other alterations in their phenotypes that incur potential positive changes compared to the wild type strain. There are three closely spaced locations within cluster I of the *rpoB* gene that are associated with mutations conferring rifampicin resistance (Maughan et al. 2004). Each mutation has been reported to exert dramatically different effects on phenotype such as spore germination. Though the effects of mutations to the *rpoB* gene seem to induce detrimental effects to activities such as sporulation and germination, this is not always the case. In a study conducted by Inaoka et al. (2003) it was found that certain mutations to the *rpoB* gene in a *B. subtilis* strain resulted in a dramatic increase in antibiotic production. Considering this, it is possible that while the rifampicin-resistant mutant strains of GB126 might possess defects regarding to sporulation, they may also express enhanced antibiotic activity. Should this be the case, the GB126 rif strains may have some advantages when used as a biocontrol agent compared to the wild type strain. However, further tests would be needed in order to test this hypothesis, including determining the specific mutation(s) present in the *rpoB* gene within the rif-resistant strains, and evaluating their biocontrol activity.

### **Optimization of Root Printing**

The intention of “root printing” sections of root tissue from corn, cotton, and soybean inoculated with GB126 rif was to visualize where colonization was taking place. Misaghi (1990) reported that root printing allows rapid qualitative screening of bacterial isolates by direct observation. Ideally, growth of GB126 rif colonies would occur from the printed root sections with the density of colonization relating to where GB126 rif was most concentrated on each

particular section printed. Accordingly, the overall aim of optimizing root printing was to establish a pattern of colonization among the roots of corn, cotton, and soybean.

Initial testing using root printing proved challenging since differentiation of bacterial colonies of GB126 rif from native bacterial colonies could not be established. Root sections were at first printed onto TSA and TSArif with TSArif serving as a means of isolating GB126 rif from native bacteria present in the planting medium. The first experiment proved to be inconclusive because GB126 rif could not be definitively distinguished from other bacterial colonies in either the roots printed onto TSA or TSArif. What was unexpected was bacterial growth other than GB126 rif occurring on plates containing rifampicin, the antibiotic to which the GB126 mutants were created to be resistant against. The growth of other bacteria on the TSArif plates suggested that the rifampicin present had degraded to the point that it no longer possessed antibiotic properties. A couple of possibilities exist as to why the rifampicin present was ineffective. One possibility is that the rifampicin stock used in creating the agar designed for isolation was out dated and had lost its antibiotic properties. Another possibility is that the rifampicin was denatured during the process of making the agar plates for printing. This could have occurred by adding the rifampicin before the agar had cooled adequately enough once it was removed from the autoclave. In either case, the initial root printing experiment showed that rifampicin was necessary to isolate GB126 rif from printed roots by inhibiting susceptible native bacteria. Rifampicin was used from this point on as the basis of isolating GB126 rif. The old rifampicin stock was discarded and a fresh stock made to ensure age of the stock was not a factor as to why inhibition of susceptible bacteria did not occur.

During the second root printing experiment, sections of root tissue were printed onto either TSArif or TSArif containing fungicides. The purpose of using fungicides in addition to

rifampicin was to inhibit fungal growth occurring on the printed root sections in order to clearly observe potential colonization of GB126 rif. The results from this test showed that GB126 rif colonies could be observed near printed root sections, though these observations were made only on TSArif plates containing no fungicides. Additionally, colonies were only observed on roots of cotton and soybean (Figure 4) and not on corn. The reason colonies could not be observed on corn was due to fungal growth obscuring the printed root sections. No GB126 rif colonization was observed on TSArif plates containing fungicides on any of the crops printed. The results of this experiment showed that TSArif was useful for isolating GB126 rif from native bacterial populations indicating that the rifampicin remained effective unlike in the first root printing experiment. However, in this experiment, fungal growth obscured observation of printed root sections. The use of fungicides proved ineffective as no GB126 rif growth was observed on plates containing the fungicides. A change in fungicide formulation would be required.

In the third experiment using root printing of corn, cotton, and soybean, a new agar formulation was used involving different fungicides. By eliminating ipconazole and introducing thiram in the fungicide regimen, GB126 rif growth was no longer inhibited on the TSArif plates. The results of this experiment definitively showed GB126 rif colonization in all three crops tested (Figure 5). This is in contrast to the second experiment in which colonization was only observed on TSArif plates containing no fungicides. It appeared that the fungicide ipconazole, used in previous experiments, was somehow inhibiting the growth of GB126 rif. By changing the fungicide formulation to remove ipconazole and replace it with thiram, observations of colony growth could be observed. In addition, the fungicides inhibited the growth of fungi present to the extent that GB126 rif colonies could be observed before fungal growth expanded beyond the printed roots. Not only did this experiment show that GB126 rif could be isolated from crop

roots 4 WAP, it also established the use of thiram and metalaxyl as the standard fungicides to be used in preparing GB126 rif isolation media. With this change in the fungicides used, colonization could be established on root sections of corn which previously would be covered by fungal growth by the time observations were taken. From this experiment, a fungicide regimen compatible with GB126 rif growth was established and would be used in further tests.

While root printing facilitates the determination of *in situ* spatial distribution of bacterial populations on the rhizoplane of plant roots (Stanghellini and Rasmussen 1989), it does not unambiguously distinguish between colonization of the rhizosphere and colonization of the rhizoplane. Evidence that growth of both rhizosphere and rhizoplane bacteria is detected with root printing can be seen in the images taken at 4 WAP (Figure 5) where GB126 rif colonies were observed not just in proximity to the imprinted root pieces but throughout the entire agar plate. Such colonies most likely originated from residual rhizosphere soil particles that fell onto the agar plate while the root sections were being transferred and placed onto the agar. Similar results were obtained by Stanghellini and Rasmussen (1989) when preprinting was conducted on the sticky surface of masking tape prior to printing onto agar. This preprinting eliminated the origin of such colonies. With this in mind, root printing is a viable technique to acquiring colony growth visually though limitations to the technique do exist.

### **Dilution Plating for Quantifying Internal and External Root Colonization**

The purpose of dilution plating of GB126 rif from the roots of corn, cotton, and soybean was to enumerate population sizes using different sampling techniques. Dilution plating offers a means of quantifying bacterial densities with regards to colonization (Simons et al. 1996, Gamalero et al. 2004, Koele et al. 2009). Previously, dilution plating could not be performed due

to inhibition of GB126 rif growth of the initial medium used for isolation. The original medium contained the fungicide ipconazole which inhibited the growth of GB126 rif. A new fungicide formulation was prepared which replaced ipconazole with the fungicide thiram. This new formulation did not possess the same inhibitory properties that the original formulation had and enabled dilution plating to be performed.

To test the utility of the new isolation medium to quantify colonization by GB126 rif, two sampling methods were used on the roots of corn, cotton, and soybean. The two sampling methods used involved either washing the roots of each crop with tap water at the PSRC (“rhizoplane”) or simply shaking excess soil off after extracting the roots leaving behind some soil residue (“rhizosphere”). Dilution plating was performed after sampling and plates were observed for colony formation after incubating at 28.0°C for 48 h. Populations of GB126 rif could be enumerated in all three crops using both the “rhizoplane” and “rhizosphere” methods of sampling. For the remainder of the initial greenhouse testing, dilution plating was performed to better understand population sizes in corn, cotton, and soybean.

Further evaluation of dilution plating for enumerating population densities of GB126 rif was performed on soybean at 8 WAP. Several sampling methods were implemented to better understand possible locations of colonization throughout the root system. Four sampling methods were used which included sampling from the rhizosphere, the rhizoplane, the rhizosphere and endorhiza, and the endorhiza only. Among the areas of the root system sampled, populations of GB126 rif were highest from the rhizoplane area (mean 7.17 log CFU/g) and lowest in the endorhiza (mean 4.75 log CFU/g) (Table 2). The finding that populations on the rhizoplane were statistically equivalent to the rhizosphere populations was surprising and suggests that GB126 is well adapted to colonizing the rhizoplane. This is in contrast to other studies investigating

bacterial colonization of the rhizosphere and rhizoplane which generally find populations of  $10^7$ - $10^9$  CFU/g in the rhizosphere and  $10^5$ - $10^7$  CFU/g on the rhizoplane (Benizri et al. 2001).

Another interesting finding was that GB126 rif can colonize inside roots, even though populations are less inside roots than on the root surface. The results of sampling soybean roots at 8 WAP were comparable to those found in corn and cotton at 10 WAP. Populations of GB126 rif were detectable in both crops on the rhizoplane and in the endorhiza at this sampling time (Tables 3 and 4). Comparing the populations found on the rhizoplane and in the endorhiza on soybean at 8 WAP and corn and cotton at 10 WAP, those on the rhizoplane were higher than in the endorhiza. The reduced population density observed in the current study with GB126 inside roots compared to on the rhizoplane agrees with past studies of endophytes. For example, Quadt-Hallmann et al. (1997) reported that *Pseudomonas fluorescens* strain 89B-61 colonized the rhizoplane of cotton at population levels of  $8.7 \times 10^5$  CFU/g, but inside roots, the population was more than 2.5 log units lower ( $1.1 \times 10^3$  CFU/g). Despite the lower populations found in the endorhiza compared to the rhizoplane, GB126 rif was detectable at both 8 and 10 WAP demonstrating its persistence for a substantial time following seed treatment.

The final experiment in the greenhouse colonization trials involved sampling from the rhizoplane and endorhiza in all three crops at 12 WAP. It was surprising to note that at 12 WAP populations were greater in the endorhiza than on the rhizoplane with both corn and cotton. This is in contrast to previous sampling times, as well as studies performed by other authors in which the endorhiza populations are lower than those found on the rhizoplane (Quadt-Hallmann et al. 1997, Andreote et al. 2009). Results revealed that GB126 colonizes the rhizoplane and the endorhiza for at least 12 weeks. Colonization of the endorhiza is particularly important because inside the plant root, GB126 would potentially have more capacity to elicit changes in the plant

that could lead to control of nematodes. This is because when the bacterium is inside the root, it could produce signals for plant defense that could immediately alter plant gene expression, a process known as induced systemic resistance (ISR) (Kloepper and Ryu 2006). In contrast, bacterial-produced signals outside the plant in the rhizosphere could be subject to break-down by other rhizosphere microorganisms.

### **Comparative Colonization of Soybean in Acidic Soils**

To assess the capability of GB126 rif to colonize roots in acidic soils, root printing of soybean was performed. Unlike root printing methods that had been used in previous experiments, whole root systems, rather than sections, were printing in an attempt to visualize the spatial pattern of colonization on seedlings. Additionally, instead of the roots being placed on top of the agar medium, the roots were placed beneath the surface of the semi-solid agar in order to prevent fungal growth from occurring on parts of roots that can be raised above the agar surface of TSA-rif plates with the normal concentration of agar. The finding that colonization was noted in all five soil types (Figures 7-9), representing pH values ranging from 5.05 to 7.05, clearly indicates that the capacity of GB126 to grow on roots is not limited to soils at alkaline pH values. In fact, colonization of the Sand Mountain soil was particularly strong (Figure 8), and this soil had a pH value of 5.05.

### **Microplot Trials to Confirm Colonization Capacity**

Microplot trials were performed using GB126 rif in order to verify the bacterium's capacity to colonize the roots, as seen in greenhouse testing, of various plants and in varying soil conditions. Two trials were conducted, one in which GB126 rif was applied to three different

crops and one in which a single crop was used in five different soil types. The purpose of utilizing microplot trials was to study colonization in conditions that would more closely mimic those found in the field.

The first microplot trial involved determining if GB126 rif was capable of colonizing the roots of different crops as had been seen in greenhouse testing. The crops used in this experiment were corn, cotton, and soybean. By performing dilution plating of the rhizoplane and endorhiza, it was determined that GB126 rif could be isolated from all three crops at 3, 6, and 12 WAP (Figures 10-12). The results of the first microplot trial suggest that GB126 is capable of colonizing different crops in conditions that are closely related to those one would experience in field plots. More importantly, the findings propose that GB126 is capable of colonizing multiple plant types as opposed to being restricted to a single crop. This is supported by past studies involving inoculation of several different crops with PGPR strains such as one conducted by Höflich et al. (1995). In their study, the authors were able to recover two PGPR strains, *Pseudomonas* strain PsIA12 and *Rhizobium leguminosarum* bv. *trifolii* strain R39, from the rhizosphere of wheat, corn, rape, oil radish, pea, alfalfa, and sugar beet. The significance of being able to colonize several different crops, as opposed to being restricted to a single crop, is that the utility of a PGPR strain is enhanced. Considering GB126 rif was recovered from three different crops, the findings from the first microplot trial suggest it can be applied to a variety of plant types and colonize successfully.

During the second microplot trial, GB126 rif was tested for its ability to colonize plant roots in various soil conditions. For this experiment, soybean was chosen as the crop to test colonizing in five different soil types. To determine colonization of GB126 rif in this microplot trial, dilution plating was performed in the same manner as the first microplot trial. The results

from this trial revealed that GB126 rif was able to be recovered from both the rhizoplane and endorhiza from the different soil types tested 3, 6, and 12 WAP (Figures 13-17). The except to this was isolating GB126 rif from the endorhiza of the Piedmont soil type. Colonies could not be isolated from the endorhiza at any of the sampling periods. While many possibilities exist as to the cause of not being able to isolate GB126 rif from the Piedmont soil, one potential reason for failure to obtain colonies could be due to a phenomenon known as ‘rifampicin-masking’.

Masking of rifampicin-resistant marked mutants, as described by McInroy et al. (1995), occurs when rifampicin-resistant bacteria fail to grow on media containing rifampicin when placed directly onto such media. The authors observed this phenomenon when investigating rifampicin-resistant bacteria on cucumber and discovered that internal colonization could only be observed if bacteria were plated onto TSA without rifampicin present. The results from their investigation suggest that rifampicin-resistance may be altered *in planta* which may affect isolation attempts.

While complications arose with isolating GB126 rif from the single soil type, populations were enumerated for both microplot trials. The most important finding from both microplot trials was that GB126 colonizes roots of different crops and in different soil types in conditions outside of the greenhouse. Populations were isolated from both the rhizoplane and endorhiza showing that GB126 forms a very close association with the roots. Populations of GB126 rif in the microplot trials generally declined over time, a finding that is in agreement with other studies. For example, Shishido et al. (1995) reported that rhizosphere populations of *Bacillus polymyxa* strains L6-16R and Pw-2R on pine seedlings were greater than  $5 \times 10^7$  CFU/g at 2 weeks after planting compared to  $5 \times 10^6 - 5 \times 10^7$  CFU/g at 4 weeks. Declining population densities of PGPR strains was also described by Andreote et al. (2009) when investigating *Pseudomonas putida* strain P9R on potato at different growth stages. The authors found that at growth stage 1,

rhizoplane populations of P9R on one cultivar of potato were 4.99 log CFU/g while at growth stage 6 the population had fallen to 4.22 log CFU/g. Similarly, the population of P9R of the endosphere (endorhiza) at growth stage 1 was 2.09 log CFU/g compared to 0.77 log CFU/g at growth stage 6. While both of these studies indicate a decline in PGPR populations over time, bacteria populations were still recoverable even past extended periods after inoculation. From the results of the microplot trials conducted with GB126 rif, it appears that populations of the bacterium are present even at 12 WAP, indicating that GB126 is capable of colonizing roots after an extended time post inoculation.

## VI. REFERENCES

- Agrios, George, N. 2005. In: Plant Pathology, fifth ed. Elsevier Academic Press, Burlington, pp. 888.
- Ahmad, F., Ahmad, I., and Khan, M.S. 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiological Research*, 163:173-181.
- Andreote, F.D., de Araújo, W.L., de Azevedo, J.L., van Elsas, J.D., da Rocha, U.N., and van Overbeek, L.S. 2009. Endophytic colonization of potato (*Solanum tuberosum* L.) by a novel competent bacterial endophyte, *Pseudomonas putida* strain P9, and its effect on associated bacterial communities. *Appl. Environ. Microbiol.* 75(11):3396-3406.
- Babalola, Olubukola, Oluranti. 2010. Beneficial bacteria of agricultural importance. *Biotechnol Lett.* 32:1559-1570.
- Bashan, Y., and Holguin, G. 1998. Proposal for the division of plant growth-promoting rhizobacteria into two classifications: biocontrol-PGPB (plant growth-promoting bacteria) and PGPB. *Soil Biol. Biochem.* 30, 1225-1228.
- Beattie, G.A. and Lindow, S.E. 1995. The secret life of foliar bacterial pathogens on leaves. *Annu. Rev. Phytopathol.* 33:145-72.
- Bressen, W. and Borges, M.T. 2004. Delivery methods for introducing endophytic bacteria into maize. *BioControl* 49:315-322.
- Buddrus-Schiemann, K, Schmid, M., Schreiner, K., Welzl, G., and Hartmann, A. 2010. Root colonization by *Pseudomonas* sp. DSMZ 13134 and impact on the indigenous rhizosphere bacterial community of barley. *Microb. Ecol.* 60:381-393.
- Carroll, G. 1988. Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. *Ecology* 69:2-9.
- Compant, S., Reiter, B., Sessitsch, A., Nowak, J., Clément, C., and Ait Barka, E. 2005. Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. *Appl. Environ. Microbiol.* 71(4):1685-1693.
- Davis, C.L. and Brlansky, R.H. 1991. Use of immunogold labeling with scanning electron microscopy to identify phytopathogenic bacteria on leaf surfaces. *Appl. Environ. Microbiol.* 57(10):3052-3055.

- Gamalero, E., Lingua, G, Capri, F. G., Fusconi, A, Berta, G, and Lemanceau, P. 2004. Colonization pattern of primary tomato roots by *Pseudomonas fluorescens* A6RI characterized by dilution plating, flow cytometry, fluorescence, confocal and scanning electron microscopy. *FEMS Microbiol. Ecol.* 48:79-87.
- Hallman, J., Quadt-Hallmann, A., Mahaffee, W.F., and Kloepper, J.W. 1997. Bacterial endophytes in agricultural crops. *Can. J. Microbiol.*, 43:895-914.
- Hirano, S.S. and Upper, C.D. 1983. Ecology and epidemiology of foliar bacterial plant pathogens. *Ann. Rev. Phytopathol.*, 21:243-269.
- Höflich, G., Wiehe, W., and Hecht-Buchholz, C. 1995. Rhizosphere colonization of different crops with growth promoting *Pseudomonas* and *Rhizobium* bacteria. *Microbiol. Res.* 150:139-147.
- Inaoka, T., Takahashi, K., Yada, H., Yoshida, M., and Ochi, K. 2003. RNA polymerase mutation activates the production of a dormant antibiotic 3,3'-neotrehalosadiamine via an autoinduction mechanism in *Bacillus subtilis*. *J. Biol. Chem.*, 279(5):3885-3892.
- Klopper, J.W. and Schroth, M.N. 1978. Plant growth-promoting rhizobacteria on radishes. In: *Station de Pathologie Végétale de Phytobactériologie (Ed.), Proceedings of the 4<sup>th</sup> International Conference on Plant Pathogenic Bacteria.* vol. II. Gilbert-Clarey, Tours, France, pp. 879-882.
- Kloepper, J.W., Schroth, M.N., and Miller, T.D. 1980. Effects of rhizosphere colonization by plant-growth promoting rhizobacteria on potato plant development and yields. *Phytopathology*, 70:1078-1082.
- Kloepper, J.W. Schippers, B., and Baker, P.A.H.M. 1992. Proposed elimination of the term endorhizosphere. *Phytopathology*, 82:726-727.
- Kloepper, J.W. and Ryu, C-M. 2006. Bacterial endophytes as elicitors of induced systemic resistance. *Microbial Root Endophytes*, 33-52. Springer-Verlag, Berlin.
- Koele, N., Turpault, M., Hildebrand, E.E., Uroz, S., and Frey-Klett, P. 2009. Interactions between mycorrhizal fungi and mycorrhizosphere bacteria during mineral weathering: budget analysis and bacterial quantification. *Soil Biol. Biochem.* 41:1935-1942.
- Mahaffee, W.F. and Kloepper, J.W. 1997. Temporal changes in the bacterial communities of soil, rhizosphere, and endorhiza associated with field-grown cucumber (*Cucumis sativus* L.). *Microb Ecol.*, 34:210-223.
- Malakoff, D. 1998. Coastal ecology: death by suffocation in the Gulf of Mexico. *Science*, 281:190-192.

- Mariano, R.L.R. and McCarter, S.M. 1993. Epiphytic survival of *Pseudomonas viridiflava* on tomato and selected weed species. *Microb. Ecol*, 26:47-58.
- Maughan, H., Galeano, B., and Nicholson, W.L. 2004. Novel *rpoB* mutations conferring rifampin resistance on *Bacillus subtilis*: global effects on growth, competence, sporulation, and germination. *J. Bacteriol*, 186(8):2481-2486.
- Misaghi, I. J. 1990. Screening bacteria for root colonizing ability by a rapid method. *Soil Biol. Biochem*, 22(8):1085-1088.
- Moeller, R., Vlašić, I., Reitz, G., and Nicholson, W.L. 2012. Role of altered *rpoB* alleles in *Bacillus subtilis* sporulation and spore resistance to heat, hydrogen peroxide, formaldehyde, and glutaraldehyde. *Arch Microbiol*, 194:759-767.
- Nautiyal, C. Shekhar. 1997. Rhizosphere competence of *Pseudomonas* sp. NBRI9926 and *Rhizobium* sp. NBRI9513 involved in the suppression of chickpea (*Cicer arietinum* L.) pathogenic fungi. *FEMS Microbiol. Ecol*, 23:145-158.
- Quadt-Hallmann, A., Hallmann, J., and Kloepper, J.W. 1997. Bacterial endophytes in cotton: location and interaction with other plant-associated bacteria. *Can. J. Microbiol*, 43:254-259.
- Romanstchuk, Martin. 1992. Attachment of plant pathogenic bacteria to plant surfaces. *Annu. Rev. Phytopathol*, 30:225-243.
- Rothballer, M., Eckert, B., Schmid, M., Fekete, A., Schloter, M., Lehner, A., Pollmann, S. and Hartmann, A. 2008. Endophytic root colonization of gramineous plants by *Herbaspirillum frisingense*. *FEMS Microbiol. Ecol*. 66:85-95.
- Ryan, R. P., Germaine, K., Franks, A., Ryan, D.J., and Dowling, D.N. 2008. Bacterial endophytes: recent developments and applications. *FEMS microbiology letters*. 2008 Jan. 278(1) p. 1-9.
- Shishido, M., Loeb, B.M., and Chanway, C.P. 1995. External and internal root colonization of lodgepole pine seedlings by two growth-promoting *Bacillus* strains originated from different root microsites. *Can. J. Microbiol*, 41:707-713.
- Simons, M., van der Bij, A.J., Brand, I., de Weger, L.A., Wijffelman, C.A., Lugtenburg, B.J.J. 1996. Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting *Pseudomonas* bacteria. *Molecular Plant-Microbe Interactions* 9(7):600-607.
- Stanghellini, M.E. and Rasmussen, S.L. 1989. Root printing: A technique for the determination of the in situ spatial distribution of bacteria on the rhizoplane of field-grown plants. *Phytopathology* 79:1131-1134.

- Sturz, A.V. 1995. The role of endophytic bacteria during seed piece decay and potato tuberization. *Plant and Soil*, 175:257-263.
- Sturz, A.V., Christie, B.R., and Nowak, J. 2000. Bacterial endophytes: potential role in developing sustainable systems of crop production. *Critical Reviews in Plant Sciences*, 19:1-30.
- Timmer, L.W., Morris, J.J., and Achor, D. 1987. Growth and survival of Xanthomonads under conditions nonconducive to disease development. *Phytopathology* 77:1341-1345.
- Timmusk, S., Grantcharova, N. and Wagner, E.G.H. 2005. *Paenibacillus polymyxa* invades plant roots and forms biofilms. *Appl. Environ. Microbiol*, 71(11):7292-7300.
- Vidwans, S. J., Ireton, K., and Grossman, A.D. 1995. Possible role for the essential GTP-binding protein Obg in regulating the initiation of sporulation in *Bacillus subtilis*. *J. Bacteriol*, 177(11): 3308-3311.

## VII. TABLES

### Dilution Plating for Quantifying Internal and External Root Colonization

**Table 1. Population densities of GB126 rif at 6 WAP**

<u>Crop</u>	<u>Sampling method</u>	<u>Log CFU/g<sup>c</sup></u>
Corn	Rhizoplane <sup>a</sup>	5.58
	Rhizosphere <sup>b</sup>	5.31
Cotton	Rhizoplane	6.25
	Rhizosphere	6.81
Soybean	Rhizoplane	6.08
	Rhizosphere	5.71

<sup>a</sup>Rhizoplane = roots washed using tap water at PSRC (root surface)

<sup>b</sup>Rhizosphere = roots shaken to remove excess soil (residual soil particles attached)

<sup>c</sup>Plating medium = 50% TSA, pH 8.0 + rifampicin (100 µg/ml), metalaxyl (100 µg/ml), and thiram (25 µg/ml)

**Table 2. Population densities of GB126 rif at 8 WAP on soybean<sup>a</sup>**

<u>Sampling method</u>	<u>Log CFU/g<sup>f*</sup></u>
Rhizosphere <sup>b</sup>	6.37 AB; ab
Rhizoplane <sup>c</sup>	7.17 A; a
Rhizosphere + endorhiza <sup>d</sup>	5.20 AB; bc
Endorhiza <sup>e</sup>	4.75 B; c
LSD <sub>0.01</sub>	6.37
LSD <sub>0.05</sub>	7.17

<sup>a</sup>Means of 6 replicates

<sup>b</sup>Rhizosphere = roots shaken to remove excess soil (residual soil particles attached)

<sup>c</sup>Rhizoplane = roots washed using tap water at PSRC (root surface)

<sup>d</sup>Rhizosphere + endorhiza = roots shaken to remove excess soil followed by grinding

<sup>e</sup>Endorhiza = roots subjected to surface disinfestation and grinding

<sup>f</sup>Plating medium = 50% TSA, pH 8.0 + rifampicin (100 µg/ml), metalaxyl (100 µg/ml), and thiram (25 µg/ml)

\*Means within a column that are followed by a different capital letter are significantly different from each other at  $P = 0.01$ . Smaller case letters indicate differences at  $P = 0.05$ .

**Table 3. Population densities of GB126 rif at 10 WAP on corn<sup>a</sup>**

<u>Sampling method</u>	<u>Log CFU/g<sup>d*</sup></u>
Rhizoplane <sup>b</sup>	4.87 A; a
Endorhiza <sup>c</sup>	4.10 A; b
LSD <sub>0.01</sub>	0.86
LSD <sub>0.05</sub>	0.57

<sup>a</sup>Means of 4 replicates.

<sup>b</sup>Rhizoplane = roots washed using tap water at PSRC (root surface)

<sup>c</sup>Endorhiza = roots subjected to surface disinfestation and grinding

<sup>d</sup>Plating medium = 50% TSA, pH 8.0 + rifampicin (100 µg/ml), metalaxyl (100 µg/ml), and thiram (25 µg/ml)

\*Means within a column that are followed by a different capital letter are significantly different from each other at  $P = 0.01$ . Smaller case letters indicate differences at  $P = 0.05$ .

**Table 4. Population densities of GB126 rif at 10 WAP on cotton<sup>a</sup>**

<u>Sampling method</u>	<u>Log CFU/g<sup>d*</sup></u>
Rhizoplane <sup>b</sup>	4.72 A; a
Endorhiza <sup>c</sup>	2.70 A; a
LSD <sub>0.01</sub>	3.27
LSD <sub>0.05</sub>	2.16

<sup>a</sup>Means of 4 replicates.

<sup>b</sup>Rhizoplane = roots washed using tap water at PSRC (root surface)

<sup>c</sup>Endorhiza = roots subjected to surface disinfestation and grinding

<sup>d</sup>Plating medium = 50% TSA, pH 8.0 + rifampicin (100 µg/ml), metalaxyl (100 µg/ml), and thiram (25 µg/ml)

\*Means within a column that are followed by a different capital letter are significantly different from each other at  $P = 0.01$ . Smaller case letters indicate differences at  $P = 0.05$ .

**Table 5. Population densities of GB126 rif at 12 WAP on corn<sup>a</sup>**

<u>Sampling method</u>	<u>Log CFU/g<sup>d*</sup></u>
Rhizoplane <sup>b</sup>	3.54 A; a
Endorhiza <sup>c</sup>	4.82 B; b
LSD <sub>0.01</sub>	1.02
LSD <sub>0.05</sub>	0.67

<sup>a</sup>Means of 4 replicates.

<sup>b</sup>Rhizoplane = roots washed using tap water at PSRC (root surface)

<sup>c</sup>Endorhiza = roots subjected to surface disinfestation and grinding

<sup>d</sup>Plating medium = 50% TSA, pH 8.0 + rifampicin (100 µg/ml), metalaxyl (100 µg/ml), and thiram (25 µg/ml)

\*Means within a column that are followed by a different capital letter are significantly different from each other at  $P = 0.01$ . Smaller case letters indicate differences at  $P = 0.05$ .

**Table 6. Population densities of GB126 rif at 12 WAP on cotton<sup>a</sup>**

<u>Sampling method</u>	<u>Log CFU/g<sup>d*</sup></u>
Rhizoplane <sup>b</sup>	4.83 A; a
Endorhiza <sup>c</sup>	5.73 A; a
LSD <sub>0.01</sub>	1.93
LSD <sub>0.05</sub>	1.27

<sup>a</sup>Means of 4 replicates.

<sup>b</sup>Rhizoplane = roots washed using tap water at PSRC (root surface)

<sup>c</sup>Endorhiza = roots subjected to surface disinfestation and grinding

<sup>d</sup>Plating medium = 50% TSA, pH 8.0 + rifampicin (100 µg/ml), metalaxyl (100 µg/ml), and thiram (25 µg/ml)

\*Means within a column that are followed by a different capital letter are significantly different from each other at  $P = 0.01$ . Smaller case letters indicate differences at  $P = 0.05$ .

**Table 7. Population densities of GB126 rif at 12 WAP on soybean<sup>a</sup>**

<u>Sampling method</u>	<u>Log CFU/g<sup>d*</sup></u>
Rhizoplane <sup>b</sup>	4.99 A; a
Endorhiza <sup>c</sup>	4.82 A; a
LSD <sub>0.01</sub>	5.04
LSD <sub>0.05</sub>	3.33

<sup>a</sup>Means of 4 replicates.

<sup>b</sup>Rhizoplane = roots washed using tap water at PSRC (root surface)

<sup>c</sup>Endorhiza = roots subjected to surface disinfestation and grinding

<sup>d</sup>Plating medium = 50% TSA, pH 8.0 + rifampicin (100 µg/ml), metalaxyl (100 µg/ml), and thiram (25 µg/ml)

\*Means within a column that are followed by a different capital letter are significantly different from each other at  $P = 0.01$ . Smaller case letters indicate differences at  $P = 0.05$ .

## VIII. FIGURES

### Bacterial Strains

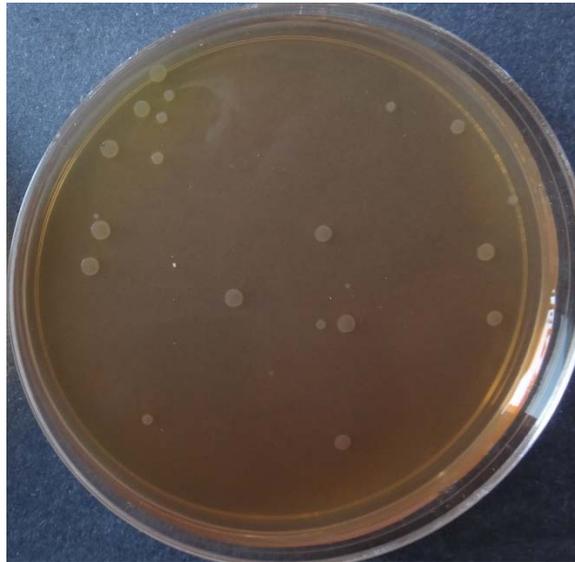


Figure 4. . Photographs of GB126 rif colonies at 48 h on 50% TSArif, pH 8.0. Colonies present are representative of the typical morphology of the rifampicin-resistant mutants of GB126.

### Optimization of Root Printing

#### Root Printing – 2 WAP

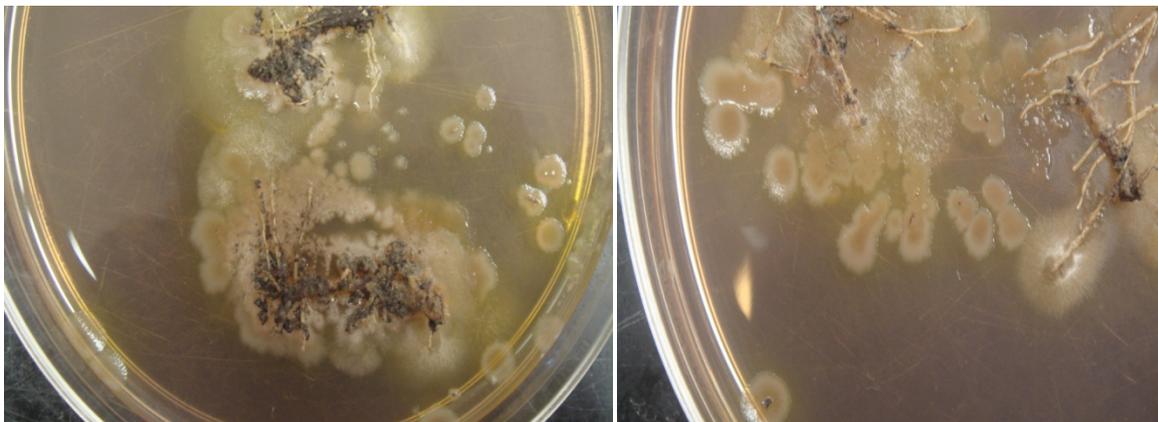


Figure 5. Photographs of root printing at 2 WAP. (Left) Soybean root sections printed onto 50% TSArif without fungicides and (right) cotton root sections printing onto the same medium.

## Root Printing – 4 WAP



Figure 6. Photographs of root printing at 4 WAP. In all images, plates on the left are root sections from GB126 rif inoculated treatments and on the right are non-inoculated controls. The crops present are corn (upper), cotton (middle), and soybean (lower). Colonies of GB126 rif can be seen throughout the plates with root sections printed onto them which were inoculated via bacterial suspension onto seeds.

## Comparative Root Colonization of Soybean in Acidic Soils

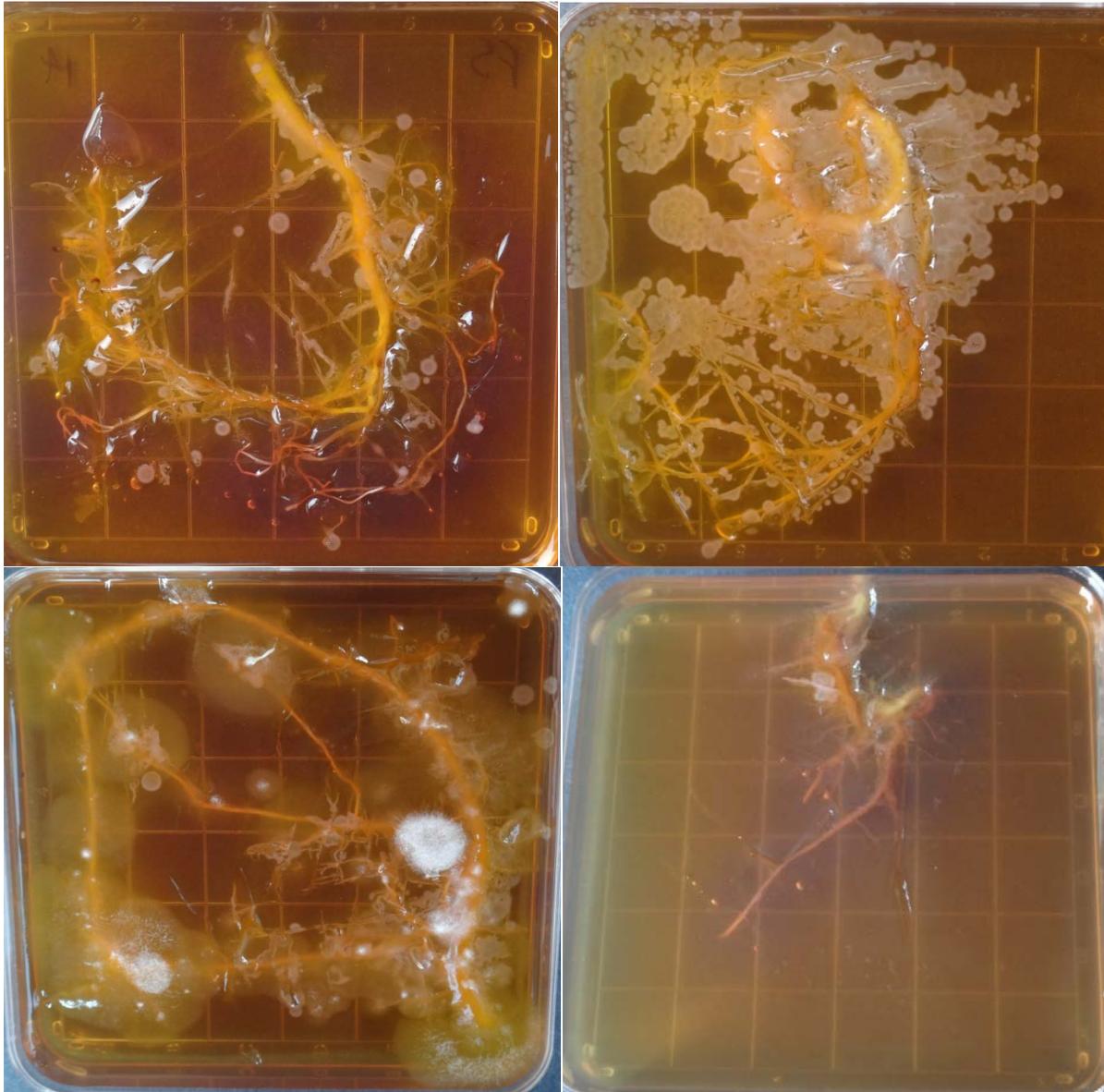


Figure 7. Photographs of root printing performed using soybean at 14 days after planting (DAP). The whole root system of each soybean plant was pressed into a semi-solid agar and incubated at 28.0°C for 48 h before photographs were taken. Soybean was planted in different soil types with different pH values. Upper two plates show roots from the “control” soil (a 1:1 field soil (clay loam) to Sunshine potting mix) (pH 7.05). Lower two plates show roots in soil from Blackbelt (31.2-40.4-28.4%) (pH 6.9).

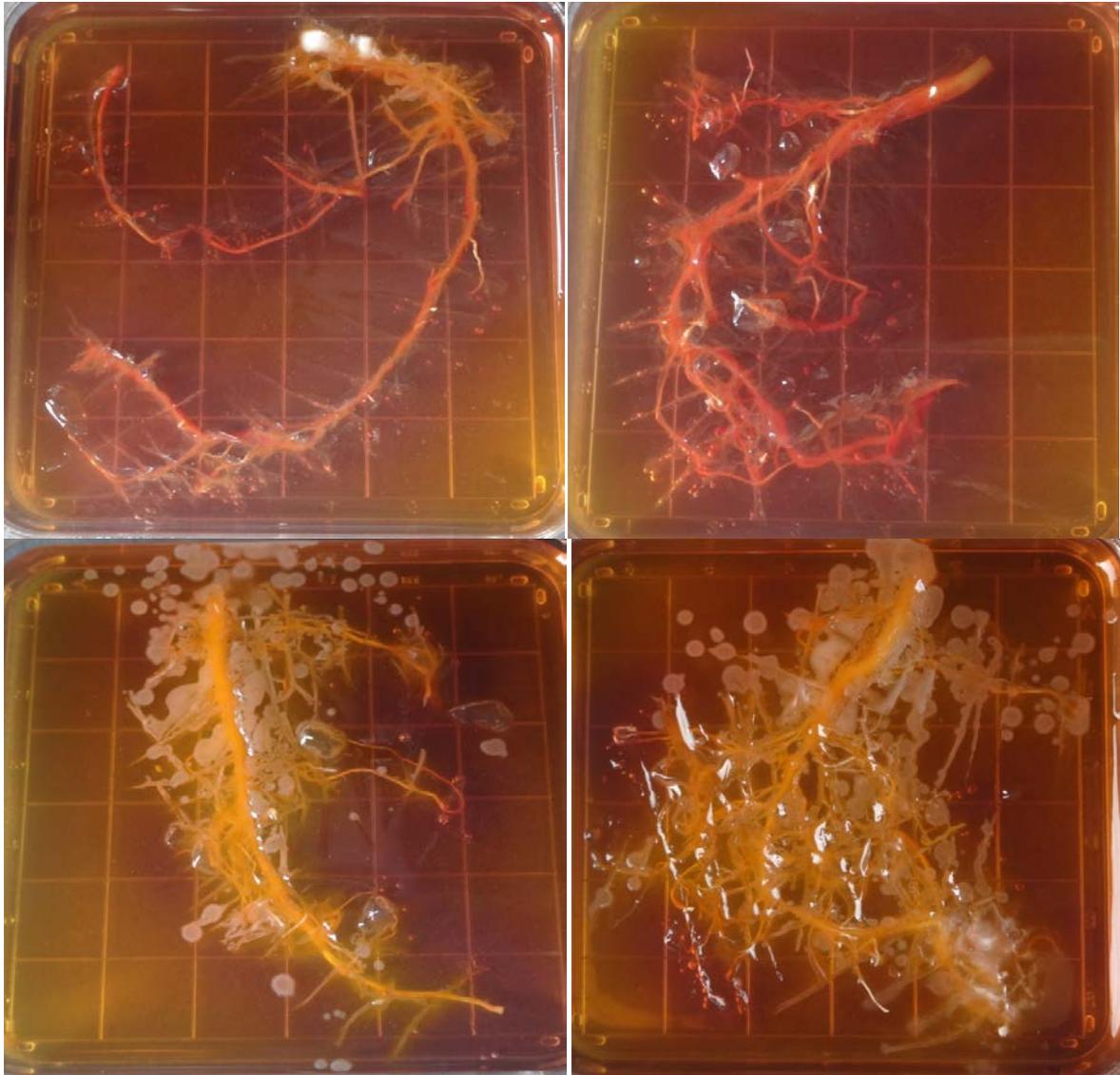


Figure 8. Additional photographs of root printing performed using soybean at 14 days after planting (DAP). Upper two plates show soybean roots from Piedmont soils (sand-clay-silt: 52-25-23%) (pH 5.15). Lower two plates show soybean roots in soil from Sand Mountain (56-12-33%) (pH 5.05).



Figure 9. Photograph of single soybean root system printed 14 DAP from Wiregrass soils (sand-clay-silt: 57-15-28%) (pH 6.15) taken 48 h post incubation at 28.0°C. This was the only soybean to germinate in the Wiregrass soils.

### Microplot Trials to Confirm Colonization Capacity as seen in Greenhouse Trials

Average log CFU/g GB126 rif populations over time – Microplot Trial 1

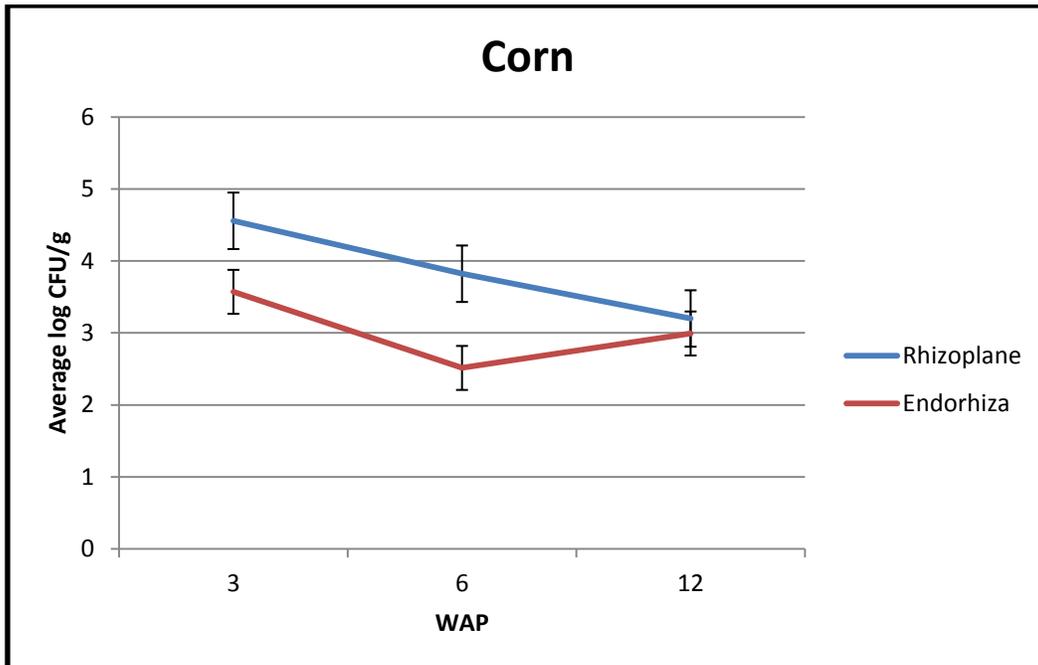


Figure 10. GB126 rif average log CFU/g populations in microplot trial 1 on corn. Populations sampled from the rhizoplane and endorhiza at 3, 6, and 12 WAP.

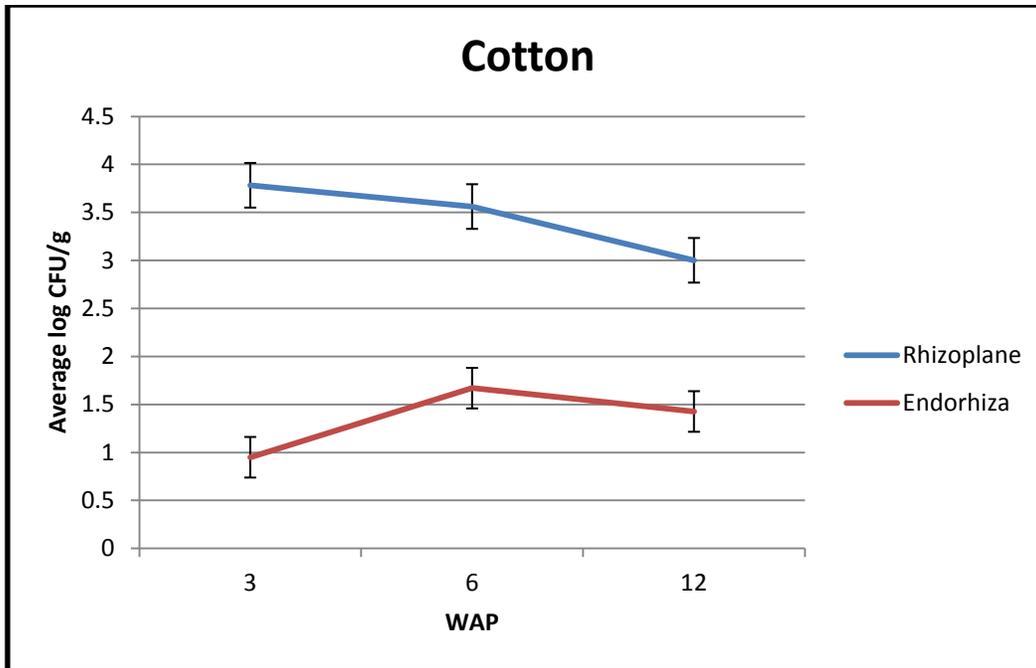


Figure 11. GB126 rif average log CFU/g populations in microplot trial 1 on cotton. Populations sampled from the rhizoplane and endorhiza at 3, 6, and 12 WAP.

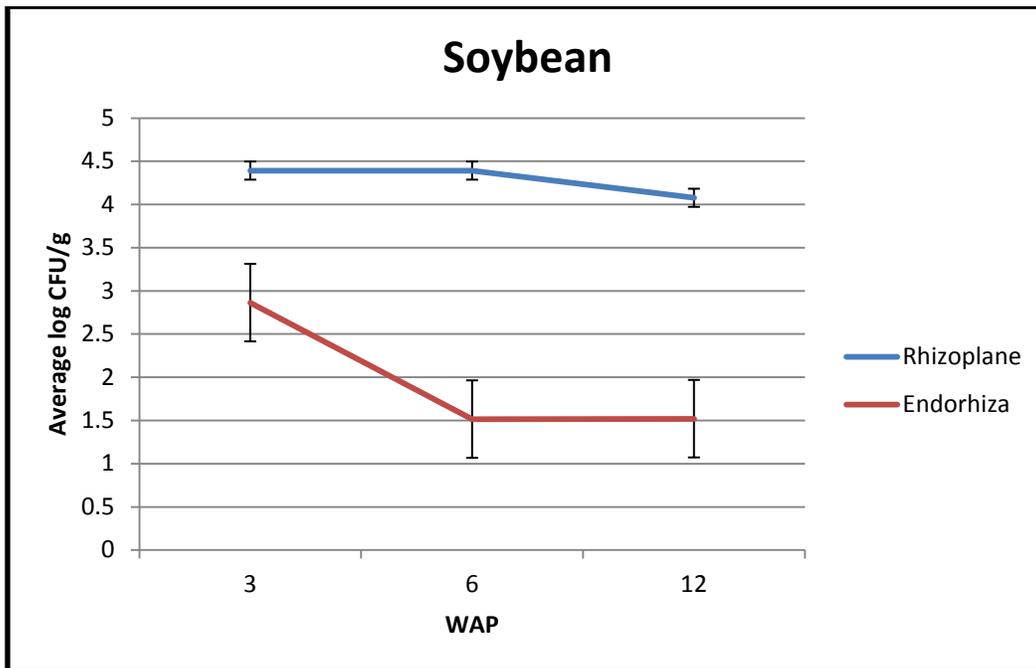


Figure 12. GB126 rif average log CFU/g populations in microplot trial 1 on soybean. Populations sampled from the rhizoplane and endorhiza at 3, 6, and 12 WAP.

Average log CFU/g GB126 rif populations over time – Microplot Trial 2

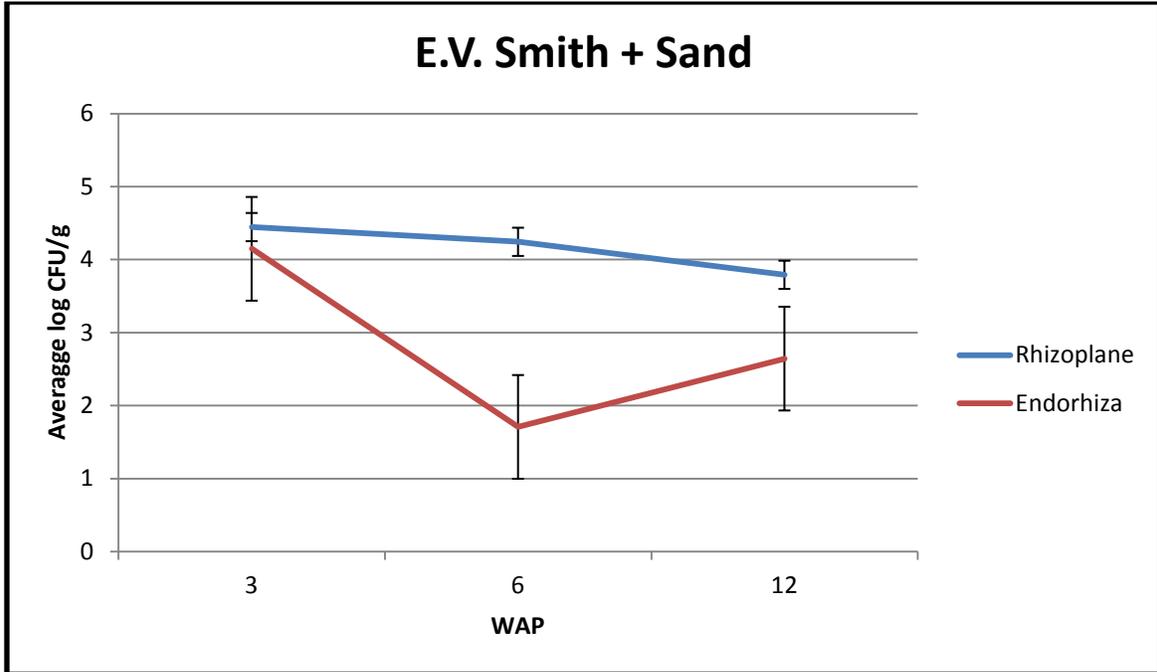


Figure 13. GB126 rif average log CFU/g populations in microplot trial 2 on soybean. The soil type used was a 3:1 ratio of field soil from E.V. Smith (sand-clay-silt: 86.8-2.4-10.8%) to sand (pH 5.8) (control soil). Populations sampled from the rhizoplane and endorhiza at 3, 6, and 12 WAP.

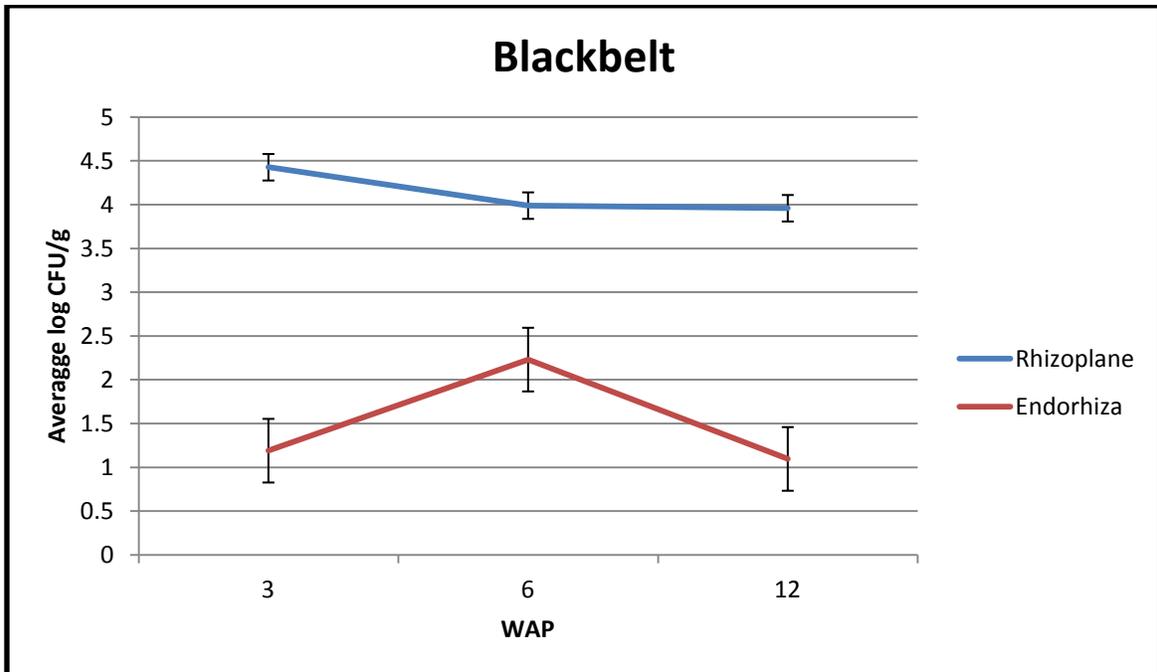


Figure 14. GB126 rif average log CFU/g populations in microplot trial 2 on soybean. The soil type used was Blackbelt (31.2-40.4-28.4%, pH 6.9). Populations sampled from the rhizoplane and endorhiza at 3, 6, and 12 WAP.

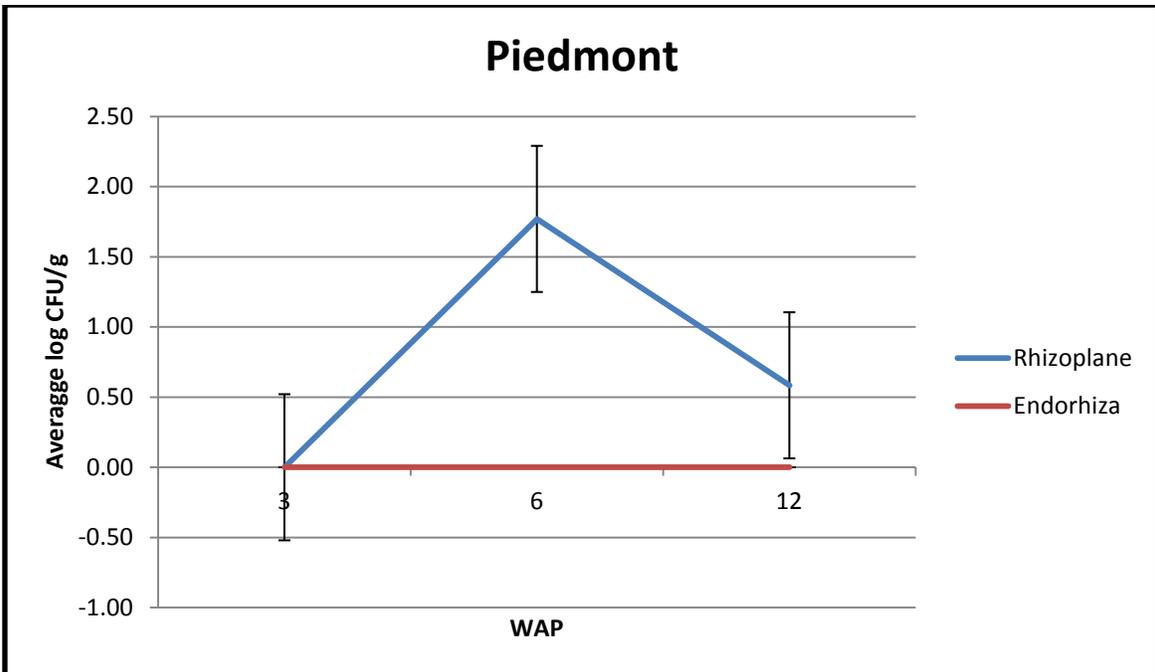


Figure 15. GB126 rif average log CFU/g populations in microplot trial 2 on soybean. The soil type used was Piedmont (52-25-23%, pH 5.15). Populations sampled from the rhizoplane and endorhiza at 3, 6, and 12 WAP.

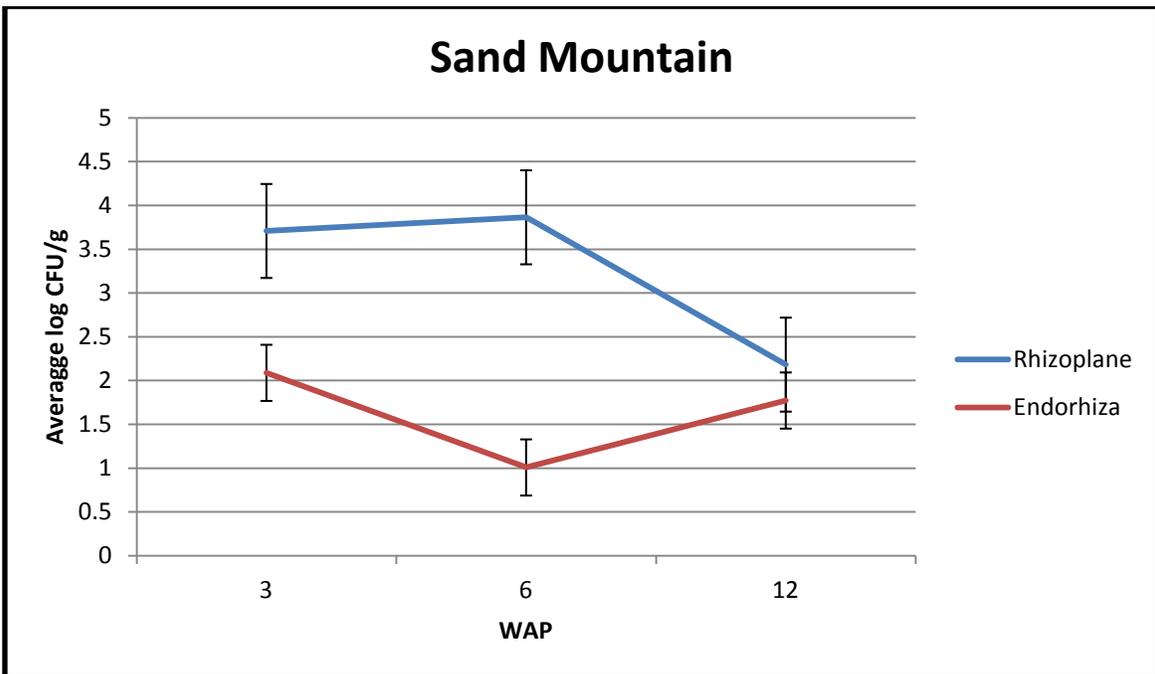


Figure 16. GB126 rif average log CFU/g populations in microplot trial 2 on soybean. The soil type used was Sand Mountain (56-12-33%, pH 5.05). Populations sampled from the rhizoplane and endorhiza at 3, 6, and 12 WAP.

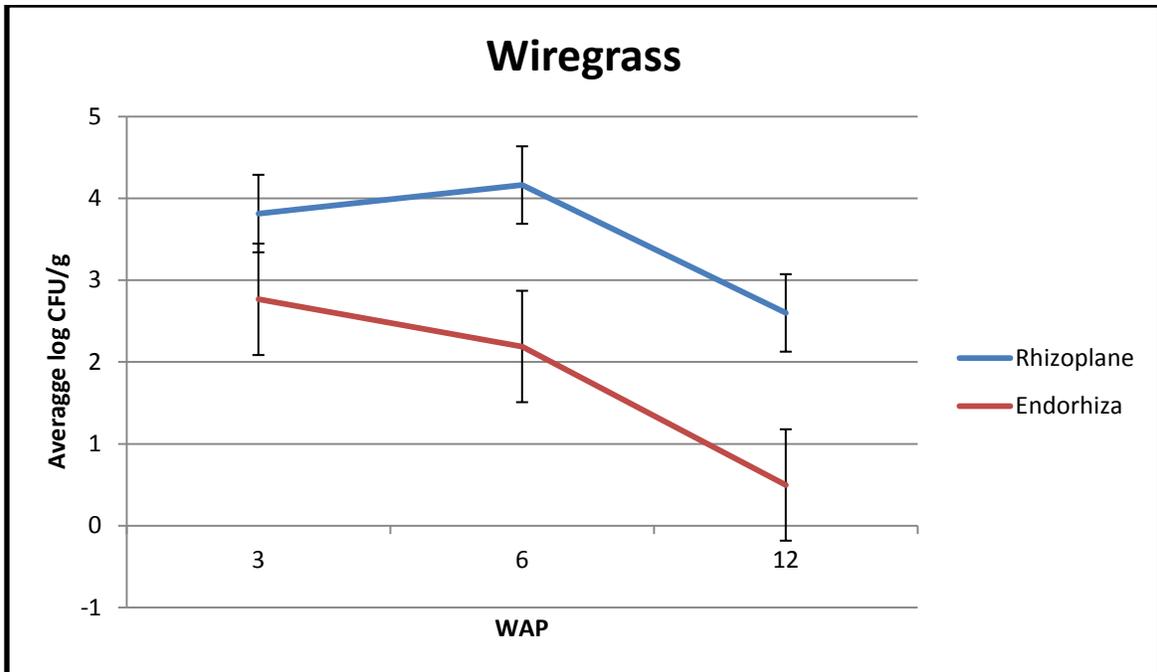


Figure 17. GB126 rif average log CFU/g populations in microplot trial 2 on soybean. The soil type used was Wiregrass (57-15-28%, pH 6.15). Populations sampled from the rhizoplane and endorhiza at 3, 6, and 12 WAP.