Evaluation of *Paenibacillus* PGPR Strains for Growth Promotion and Biocontrol of Rice Sheath Blight

by

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Abstract

A study was conducted to evaluate PGPR strains belonging to the genus *Paenibacillus* for their potential as biological control agents of rice sheath blight disease caused by *Rhizoctonia solani. Paenibacillus* strains are known to produce a wide variety of antimicrobial compounds with activity against both fungi and bacteria, which led us to hypothesize that this genus of bacteria may harbor potent biological control agents of *Rhizoctonia solani*. First, a preliminary screening of 335 *Paenibacillus* strains belonging to 56 species was conducted to identify strains with antagonistic capability to *R. solani*. In total, 21 strains belonging to the species *P. peoriae, P. jamilae,* and *P. polymyxa* exhibited antagonism. These strains were then evaluated in several *in vitro* experiments to further characterize their antagonism to *R. solani*, including an advanced antibiosis assay, a sclerotia germination assay, and a detached leaf assay. All but one of the *Paenibacillus* strains completely inhibited the germination of *R. solani* sclerotia. In addition, nine strains in total significantly reduced the average disease rating in a detached leaf assay as compared to the disease control. These nine strains were then evaluated in greenhouse disease assays for their capacity to antagonize *R. solani in planta*. Six of the evaluated nine strains had significantly lower lesion length percentages, and four of these strains (JJ-195, JJ-1580, JJ-1710, JJ-1824) also had significantly lower disease ratings compared to the disease control. *Paenibacillus* strains showing antagonistic activity were also evaluated for their ability to promote the early growth of rice; however, no significant differences were observed. In addition, the colonization dynamics of three selected strains were evaluated in the rice phyllosphere

utilizing rifampicin-resistant generated mutants. Populations of all three strains decreased rapidly over the selected time points: 0, 1 week, and 2 weeks after inoculation suggesting that these strains are not well-suited to the phyllosphere, and that a large population size was not necessary for some strains to have effective biological control of *R. solani*.

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List of Abbreviations

PGPR	Plant Growth-Promoting Rhizobacteria
TSA	Tryptic Soy Agar
RTSA	Rifampicin Tryptic Soy Agar
PDA	Potato Dextrose Agar
ShB	Sheath Blight
ISR	Induced Systemic Resistance
SAR	Systemic Acquired Resistance
IAA	Indole-3-acetic acid
ACC	1-aminocyclopropane-1-carboxylate
Rif	Rifampicin

Chapter 1: Literature Review

1. Introduction

A significant reduction in the yield of crops worldwide can be attributed to plant pathogens and their deleterious effects on plant growth (Savary et al. 2012). This reduction in yield is of concern for public food security, and applications of pesticides are frequently utilized for the control of plant pests; however, single applications of pesticides are rarely enough to effectively reduce pest populations so repeated applications are used. Public concern over the use of pesticides in agriculture has increased throughout the last few decades. While pesticides help to reduce pest and disease populations, their application can potentially lead to the development of pesticide resistance in pest populations (Hahn, 2014; Gressel, 2011), have non-target effects on other organisms (Pimental and Levitan, 1986), and contaminate land and ground water resources (Manuel et al. 2008).

Reflecting growing public interests, research into biological control agents and their commercialization have received increased attention in recent years. The focus of contemporary biological control has been largely on that of microbial inoculants to improve crop health (Gardener et al. 2002). Among these microbial inoculants are a group of beneficial, plant-associated bacteria termed plant growth-promoting rhizobacteria (PGPR), which can help to reduce the amount of pesticides used in conventional agriculture (Kloepper and Schroth, 1978). PGPR help to promote crop health through an assortment of mechanisms including increasing the nutrient status of plants, regulating the development of plants through phytohormone production, and through protection by the production of various antimicrobial compounds or by priming innate plant defenses (Figueiredo et al. 2010; Compant et al. 2010).

The genus *Paenibacillus* contains a group of PGPR with wide applications to agriculture including promotion of plant growth and suppression of plant-pathogenic organisms. *Paenibacillus* species are well known for their production of antibiotics, lytic enzymes, and other antimicrobial compounds that have activity against a variety of plant-pathogenic fungi and bacteria (Martin et al. 2003; Lee et al. 2012; Naing et al. 2014). Additionally, strains of *Paenibacillus* are frequently implicated in plant growth promotion through the production of plant phytohormones (Shokri and Emtiazi, 2010) and by assisting in nutrient acquisition through nitrogen fixation (Padda et al. 2017), phosphorus solubilization (Das et al. 2013), and the production of iron-chelating siderophores (Raza and Shen, 2010). *Paenibacillus* species also

represent a group of less-explored PGPR compared to other more characterized genera such as *Bacillus*, *Pseudomonas*, and *Burkholderia*.

2. Plant Growth-promoting Rhizobacteria (PGPR)

The soil is a dynamic environment that hosts a multitude of microbial organisms including fungi, oomycetes, nematodes, protozoa, and bacteria. Bacteria represent the most prevalent microbes in the soil with estimates as high as 10^9 bacterial cells per gram of soil (Whitman et al. 1998), with highest abundance and diversity occurring in the rhizosphere over bulk soil (Yang et al. 2017; Smalla et al. 2001). The rhizosphere is the area of soil immediately surrounding the plant root where a significant amount of photo-assimilates are exuded in the form of organic acids, amino acids, polysaccharides, phenolic compounds, sugars, as well as other secondary metabolites (Walker et al. 2003). These root exudates in turn make the rhizosphere a nutrient-rich environment that supports the proliferation of several microbial organisms. Among these rhizosphere inhabitants are a group of beneficial root-associated bacteria termed by Kloepper and Schroth in 1978 as plant growth-promoting rhizobacteria (PGPR).

2.1 Plant Growth-promoting Rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) can be defined as the soil bacteria associated with the plant root, or immediately adjoining rhizosphere that directly or indirectly promote plant growth by the secretion of numerous regulatory compounds (Ahmed et al. 2014). Bashan and Holguin (1998) proposed the division of PGPR into two categories - plant growth promoting bacteria (PGPB), to describe those beneficial bacteria that were not associated with the root of the plant (i.e. stems and leaves), and PGPR to describe those bacteria inhabiting in and around the root. It appears the term PGPB has not been widely accepted, and instead the term PGPR is used interchangeably to describe any beneficial plant growth-promoting bacteria associated with plant tissues (Vessey, 2002).

PGPR can be further broken down into the following major categories based on their mechanism of growth promotion: biofertilizers which help to increase the nutrient status of the plant, biostimulants which help to promote plant growth by the production of regulatory compounds such as plant phytohormones, or bioprotectants (biopesticides) which help to suppress plant disease caused by deleterious organisms (Figueiredo et al. 2010). Effective PGPR strains may possess one or any combination of these various mechanisms of plant growth promotion.

2.2 Mechanisms of PGPR biofertilization

Vessey defined the term biofertilizer as "a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant" (Vessey, 2002). PGPR may act as biofertilizers and increase the nutrient status of plants through a multitude of mechanisms including both symbiotic and non-symbiotic biological nitrogen fixation, phosphorus solubilization, and the production of iron-chelating siderophores (Mylona et al. 1995; Han and Lee, 2005; Kloepper et al. 1980).

2.2.1 Biological Nitrogen-Fixation

Nitrogen is the most important plant macronutrient and is often the most limiting to crop production (Agren et al. 2012). The Earth's atmosphere consists of about 78% N_2, however, it is biologically inert and unavailable to plants. Around two-thirds of atmospheric nitrogen is biologically fixed by various microorganisms into the plant usable form ammonia (NH_3) by a nitrogenase enzyme system (Kim et al. 1994). While most nitrogen-fixation is carried out by a molybdenum nitrogenase, alternative nitrogenases including vanadium and iron alone do exist (Bishop et al. 1990).

Some of the most extensively-studied nitrogen-fixing PGPR are symbiotic rhizobia species such as *Bradyrhizobium*, *Rhizobium,* and *Mesorhizobium* that are symbiotically associated with the roots of legumes (Zahran, 2001). The genus *Frankia* represents a group of diazotrophic (nitrogen-fixing) actinomycetes that are symbiotically associated with the roots of non-leguminous plants (Benson and Silvester, 1993). A range of free-living diazotrophic bacteria also exist in the soil including bacteria in the genera *Azospirillum*, *Azoarcus*, *Burkholderia*, *Bacillus*, and *Paenibacillus* (Engelhard et al. 2000; Ding et al. 2005).

Free-living diazotrophic bacteria, while not symbiotically associated with plants, may still associatively fix nitrogen in or around plant tissues, thereby increasing plant growth and potentially increasing the nitrogen content of their tissues. Inoculation of canola (*Brassica napus*) with a strain of *Paenibacillus polymyxa* significantly increased foliar nitrogen content compared to an untreated control (Puri et al. 2016). Additionally, a strain of *Azospirillum sp.* and *Bacillus sphaericus* increased the nitrogen content of banana tissues by 28-40% compared to the non-treated control in another study (Baset et al. 2010). These bacteria may occur in association

with roots, stems, or leaves with certain bacterial genera showing preferential colonization of certain tissues (Roesch et al. 2008).

2.2.2 Phosphorus Solubilization

Phosphorus, like nitrogen, is an essential plant macronutrient needed for proper plant growth and function, and is involved in many metabolically important processes such as photosynthesis, respiration, energy transfer, and the biosynthesis of important compounds needed for proper growth (Khan et al. 2010). Phosphorus, although usually abundant in soils, forms mineral complexes such as phosphates or is absorbed to oxides that become biologically unavailable for plant use (Rengel and Petra, 2005). In acidic soils phosphorus is bound to inorganic aluminum and iron mineral complexes and in alkaline soils to inorganic calcium mineral complexes (Sanyal and De Datta, 1991). A considerable amount of phosphorus is also present in the soil in the organic form phytate, which is not biologically available to plants (Osborne and Rengel, 2002).

PGPR may help to convert plant unavailable forms of phosphorous in the soil to plant available forms by solubilization through the production of organic acids that lower the pH, therefore enhancing the chelation of cations associated with the mineral complexes that bind phosphorus, or through mineralization of organic phosphorus to inorganic forms (Sharma et al. 2013). The production of organic acids by bacteria is a natural by-product of bacterial metabolism and serves to chelate the ions associated with the inorganic mineral complexes of phosphorus (Al, Ca, and Fe), or by dissolving mineral phosphorus as a result of acidic anion exchange (Trolove et al. 2003). A range of organic acids may be produced by microbes that can aid in the solubilization of inorganic phosphorus complexes in the soil including acetate, oxalate, succinate, citrate, gluconate, among others (Gyaneshwar et al. 2002).

2.2.3 Production of Siderophores

Iron plays an essential role in all living things and is integral in various cellular processes including electron transport, photosynthesis, amino acid and DNA synthesis, as well as serving as a catalyst for many enzymes for redox reactions (Manthey et al. 1994). Iron is the fourth most abundant element in the Earth's crust and is present in soils in insoluble forms such as iron oxides, hydroxides, or silicate minerals but can exist in aqueous solution in two oxidative states: Fe^{+2} and Fe^{+3} (Desai et al. 2011). Fe^{+3} is not readily usable by plants since it has poor solubility at neutral and alkaline soil pH, especially in calcareous soils where it forms oxides with $CaCO_3$

(Zhang et al. 2011). Because of this, iron deficiency is most problematic in alkaline and calcareous soils which are estimated to make up one-third of the Earth's soils (Vose, 1982).

Siderophores are low molecular weight, ferric ions produced by bacteria and fungi under iron-limiting conditions that scavenge unavailable forms of iron in the soil, making them available to the microbe (Neilands, 1995). Siderophore production has been implicated in the growth promotion of plants with the premise that PGPR growing in or around plant tissues that produce siderophores could in turn increase the iron nutrition of the plants they are inhabiting. Inoculation of iron-starved tomato plants with a strain of *Chryseobacterium* and its siderophores as well as just siderophores alone alleviated the iron stress of the affected plants and increased the iron content of plant tissues significantly compared to the negative control. The increase in iron content of the tissues also trended higher but was significantly the same as the positive control (Radzki et al. 2013). The *Pseudomonas* siderophore pseudobactin was found to be the main mechanism of growth promotion on potato in a greenhouse assay (Kloepper et al. 1980). Mutant strains deficient in production of the siderophore failed to promote the growth of potato while the wild-types maintained this ability.

2.3 Mechanisms of PGPR biostimulation

Plant growth regulators, commonly referred to as hormones, play an integral role in the growth and development of plants and also aid in the response of plants to environmental stress (Davies, 2004). PGPR may act as bio-stimulants that aid in the growth and development of plants or alter the plant's response to environmental stress through the production of plant phytohormones. PGPR produce a range of plant hormones including cytokinins, gibberellins, and auxins that can modulate the growth of plants. Additionally, they can alter how plants respond to the hormone ethylene which is implicated in plant stress responses (Glick, 2012).

The plant hormone gibberellin is important in stem elongation, induction of seed germination, and fruit setting and growth, while the hormone cytokinin is important in tissue morphogenesis, cell division, and chloroplast development (Davies, 2004). Two strains of *Bacillus* (*B. pumilis* and *B. licheniformis*) had strong growth-promoting activity when applied as cell-free culture extracts to seedlings of alder (*Alnus glutinosa*) (Gutiérrez-Mañero et al. 2001). The cell-free extracts reversed the dwarfing effect of the gibberellin inhibitor paclobutrazol through bacteria-produced bioactive gibberellins. In another study, a strain of *Acinetobacter* that produced several bioactive gibberellins also promoted the growth of cucumber, Chinese cabbage,

and crown daisy when applied as a culture filtrate (Kang et al. 2009). Several strains of PGPR have also been found to produce various cytokinins *in vitro* with the potential to affect plant growth. For example a strain of *Paenibacillus* produced the cytokinin isopentenyladenine at late stationary stage of growth (Timmusk et al. 1999), and in another study a strain of *Azotobacter vinelandii* produced three different cytokinin-active fractions tentatively identified as *trans*-zeatin, isopentenyladenosine, and isopenyladenine (Taller and Tit-Yee, 1989).

Auxin is perhaps one of the most important plant hormones as it plays a role in cell division, cell extension, tissue differentiation, seed and tuber germination, lateral and adventitious root formation, photo- and gravitropism, metabolite production, and mediates resistance to environmental stressors (Glick, 2012). It is also noteworthy that auxin plays an important role in the development of nodules in legume-rhizobia symbiosis (Grunewald et al. 2009). Inoculation of soybean plants with indole-3-acetic acid (IAA) deficient mutants of *Bradyrhizobium elkanii* resulted in the development of significantly less nodules compared to plants inoculated with the auxin-producing wild-type (Fukuhara et al. 1994). Exogenous application of IAA to the deficient mutants restored the development of root-nodule symbioses. Similarly, IAA-overproducing mutants of *Sinorhizobium meliloti* increased the nodulation of the legume *Medicago* (Pii et al. 2007). Other non-symbiotic PGPR are also known for their production of the auxin indole-3-acetic acid (IAA) and its implication in plant growth promotion. Several strains of IAA and gibberellin- producing *Pseudomonas* significantly increased total root area and total number of roots in black pepper (Paul and Sarma, 2006). This root stimulation was attributed to the plant hormones auxin and gibberellin since the strains not synthesizing these hormones failed to promote these root growth parameters. It is also worth mentioning that the high production or over-production of auxin by some PGPR can result in growth suppression as a result of auxin levels that become inhibitory (Barazani and Friedman, 1999; Xie et al. 1996).

The gaseous plant hormone ethylene is involved in many plant developmental responses such as release from dormancy, senescence and abscission, and fruit ripening; however it is also termed a “stress hormone” that is involved in the plant’s response to environmental stressors such as temperature, drought, water-logging, pathogen invasion, heavy metal toxicity, and salinity stress (Davies, 2004; Saleem et al. 2007). While ethylene is important to many developmental responses in plants, excessive accumulation of ethylene in response to stress can result in defoliation, reduced root growth, and the activation of cellular processes that are

ultimately detrimental to plant productivity (Bhattacharyya and Jha, 2012). PGPR can help to alleviate plant stress responses mediated by ethylene by producing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which catalyzes the cleavage of ACC produced by the plant (Glick et al. 1998). ACC is the immediate precursor of ethylene in the plant, so its degradation by ACC deaminase can result in levels of ethylene that are non-inhibitory to growth. A variety of PGPR producing ACC deaminase have been shown to promote growth of plants under stress conditions including drought (Zahir et al. 2008), water-logging (Liddycoat et al. 2009), heavy metal toxicity (Pandey et al. 2013), and salinity stress (Bal et al. 2013; Sarkar et al. 2018).

2.4 Mechanisms of PGPR biocontrol

A significant loss in annual crop yields worldwide can be attributed to plant diseases and is of public concern for food security (Savary et al. 2012). PGPR have been gaining momentum for their use as biopesticides to decrease disease caused by plant pathogens and increase yields, while eliminating or reducing the amount of conventional pesticides used. PGPR help to promote plant growth by suppressing or inhibiting pathogenic bacteria and fungi. PGPR-mediated biological control falls under one of two mechanisms: direct antagonism to the pathogen or indirect through the induction of systemic resistance in their plant hosts (Bhattacharyya and Jha, 2012).

2.4.1 Antagonism by PGPR

PGPR may act as direct antagonists and inhibit or suppress pathogenic organisms through the production of antimicrobial metabolites (antibiosis), through competition for resources, or by degrading toxic compounds released by deleterious organisms (Compant et al. 2005). *Bacillus* and *Pseudomonas* species have been well studied for their production of antimicrobial compounds and have been implicated in the suppression of many plant pathogens in various crops. The strain *Bacillus amyloliquefaciens* B94 strongly inhibited the growth of *Rhizoctonia solani in vitro* through the production of the cyclic lipopeptide antibiotic iturin A (Yu et al. 2002). The strain *B. amyloliquefaciens* NJN-6 reduced the growth of *Fusarium oxysporum f. sp. cubense in vitro* through the production of volatile organic compounds (VOC's) (Yuan et al. 2012). Additionally, the strain *B. subtilis* CAS15 had broad spectrum inhibition to 15 plant pathogenic fungi *in vitro* and significantly reduced the growth of *Fusarium* in pepper through the production of two siderophores (Yu et al. 2011). *Pseudomonas* species produce a range of

compounds with antimicrobial properties including pyrrolnitrin (Howell and Stipanovic, 1979), hydrogen cyanide (HCN) and 2,4-diacetylphloroglucinol (DAPG) (Lanteigne et al. 2012), various phenazines (Tambong et al. 2001), and siderophores (Kloepper et al. 1980).

The rhizosphere is a nutrient rich environment allowing for the proliferation of many microorganisms and as such, competition among indigenous microflora for resources is likely high. Therefore, aggressive colonization of the rhizosphere by PGPR can exclude the establishment of soil borne pathogens and deprive them of rhizosphere resources. Siderophores, which can contribute to the iron nutrition of plants, are also implicated in biocontrol since PGPR-produced siderophores can have higher affinity for chelating insoluble iron than those produced by other pathogenic organisms (Loper et al. 1991). The inability of some deleterious *Pseudomonas* strains to utilize siderophores produced by beneficial *Pseudomonas* strains in potato is one example of how competition for resources can inhibit the growth of pathogenic organisms in the rhizosphere (Buyer et al. 1986). Competition for both carbon and nitrogen by *Pseudomonas* spp. Ps2 and Ps5 to the fungus *Colletotrichum coccodes* was attributed to the reduction in the percentage of germination tubes on detached leaves of velvetleaf (*Abutilon theophrasti*) (Fernando et al. 1996).

The detoxification of pathogen virulence factors and degradation of pathogen signaling molecules also play roles in PGPR-mediated antagonism (Compant et al. 2005). In one experiment, the leaf scald pathogen *Xanthomonas albilineans* was controlled by a strain of *Pantoea dispersa* isolated from the tissues of sugarcane diseased with *X. albilineans* (Zhang and Birch, 1996). *P. dispersa* detoxified albicidin, the antibiotic virulence factor used by *X. albilineans*, by the production of an enzyme that detoxified albicidin. Additionally, co-inoculation with *P. dispersa* and *X. albilineans* into wounded sugarcane resulted in a 98% reduction in disease severity. In another study, an avirulent strain of *Pseudomonas solanacearum* protected tomato leaves from wilt induced by fusaric acid, a nonspecific, wilt-inducing toxin produced by many *Fusarium* species (Toyoda et al. 1988). Tomato leaf cuttings soaked in suspensions of the *P. solanacearum* strain for 2-3 hours did not produce wilt symptoms when placed into flasks containing water with a concentration of 300 µg/ml of fusaric acid.

2.4.2 Induced Systemic Resistance by PGPR

Induced resistance is a physiological state in which the plant's innate defensive capacities are heightened in response to environmental stimuli of both biotic and abiotic nature (Van Loon

et al. 1998). Two states of induced resistance have been extensively described in plants: systemic acquired resistance (SAR) and induced systemic resistance (ISR). Both forms of induced resistance can be separated based on the elicitor that induces the resistance and the subsequent regulatory pathways involved (Vallad and Goodman, 2004). In general, SAR is characterized by an accumulation of salicylic acid and pathogenesis-related proteins (PRs) in response to abiotic factors such as certain chemicals or biotically to avirulent pathogens, non-pathogens, incompatible races of pathogens, or by virulent pathogens that have stalled establishment in the plant due to unfavorable climatic conditions (Van Loon et al. 1998). ISR is elicited by PGPR and results in the priming of plant defenses, utilizing jasmonate and ethylene signaling pathways, rsulting in reduced levels of disease upon pathogen infection (Vallad and Goodman, 2003; Kloepper et al. 2004). Additionally, when testing for ISR, the PGPR strain must be spatially separated from the pathogen. For example, a PGPR applied to the roots of a plant that results in reduced levels of disease caused by a foliar pathogen.

Induction of ISR by PGPR can stimulate the expression of defense-related genes in plants involved with the synthesis of peroxidase, phytoalexins, and jasmonate production resulting in reduced levels of disease caused by plant-pathogenic organisms (Sturz and Christie, 2003). Rice plants inoculated with strains of *Pseudomonas* exhibited high levels of peroxidase expression 96 hours after challenging with the pathogen *Rhizoctonia solani* compared to treatments without bacteria where peroxidase was not expressed after 72 hours (Nandakumar et al. 2001b). The same rice plants, in addition to having heightened peroxidase expression, also had significantly higher biomass levels and significantly reduced disease severity in a greenhouse assay. In another study, tomato plants treated with a strain of *Paenibacillus polymyxa* applied as a seed treatment exhibited significantly higher levels of chitinase and β-1,3-glucanase that resulted in an 88% reduction in disease caused by *Ralstonia solanacearum* (Algam et al. 2010). Another strain of *Paenibacillus polymyxa* was found to prime the transcription of salicylic acid, jasmonate, and ethylene signaling pathways, which are involved in plant defense response, through the production of species-specific volatile organic compounds (VOC's) in *Arabidopsis* (Lee et al. 2012).

2.5. The genus *Paenibacillus*

Historically any bacterium was termed a *Bacillus* if it was rod-shaped, had aerobic or facultatively anaerobic metabolism, and formed endospores, however these bacterial

characteristics are not quite sufficient for the classification of bacteria into a single genus (Zeigler, 2013). A study in 1988 utilizing numerical taxonomy based on 188 unit characteristics suggested the framework for splitting the genus *Bacillus* (Priest et al. 1988). In 1991, a study comparing the 16S rRNA sequences of 51 *Bacillus* strains revealed that they segregated into at least five phylogenetically distinct clusters (Ash et al. 1991). One of these clusters was assigned to the novel genus *Paenibacillus* in 1993 and contained members with characteristics in common with the type strain *P. polymyxa* such as rod-shaped with gram positive wall structure, motile by means of peritrichous flagella, non-pigmented on nutrient agar, aerobic or facultatively anaerobic, and the ability to differentiate into ellipsoidal endospores that swell the sporangium (Ash et al. 1993). The genus *Paenibacillus* is derived from the latin words *paene-* meaning "almost" and *bacillus*; so "almost a *bacillus*." *Paenibacillus* is contained within the family *Paenibacillaceae* which contains seven other genera: *Aneurinibacillus, Brevibacillus, Cohnella, Oxalophagus, Thermobacillus, Fontibacillus,* and *Saccharibacillus* (Mayilraj and Stackebrandt, 2014).

3. Rice

3.1 Importance and economic overview

Rice is a semi-aquatic grass in the family *Poaceae* and was first cultivated in China or India. There are two main cultivated species of rice worldwide – African rice (*Oryza glaberrima*) and the more largely cultivated Asian rice (*Oryza sativa*) (Muthayya et al. 2014). Rice is perhaps the most important crop worldwide, as it serves as a food staple for half the world's population and can account for as high 75% of the calories consumed in some Asian diets (Khush, 2005). Around 11% of the cultivatable land on Earth is dedicated to the production of rice, with more than 90% of rice production and consumption occurring in Asia (Khush, 2005). China and India alone account for 50% of the world's rice production, and worldwide about 480 million tons of milled rice are produced annually (Muthayya et al. 2014). According to the *UN World Population Prospects* (2017) the Earth's population is expected to increase to 8.6 billion by 2030. Bangladesh, China, India, and Indonesia are among some of the countries to see the largest increases in population growth and consequently these countries are among the highest consumers of rice cereal (United Nations, 2017; Dawe et al. 2010). World rice production will need to increase by an estimated 40% by 2030 to meet the growing population demand (Khush, 2005).

3.2 Rice in the United States

Rice production in the United States is virtually restricted to 6 states: Arkansas, California, Missouri, Mississippi, Louisiana, and Texas (Snyder and Slaton, 2001). The United States along with other non-Asian producing countries including Brazil, Egypt, Madagascar, and Nigeria account for 5% of the world's rice production (Muthayya et al. 2014) with more than half of the rice produced in the U.S. being exported to the global market (USDA Economic Research Service, 2019). While rice production in the U.S. does not account for a large portion of the global supply, it still contributes a significant amount to the economies of the states producing it. The rice industry contributed over $5 billion to the U.S. economy in 2009 alone and supported over 36,000 jobs (Richardson et al. 2010). Arkansas, the largest rice producing state, contributed a total output of $1.89 billion followed closely by California with $1.79 billion in 2009 (Richardson et al. 2010).

4. Rice Sheath Blight (ShB)

4.1 Overview and economic importance

Rice sheath blight (ShB) is caused by the soilborne, basidiomycete fungus *Rhizoctonia solani* and belongs to the anastomosis group 1 intraspecific group 1A (Rush and Lee, 1983). Initial infection most often occurs during the late tillering stage of the plant on the lowermost plant sheaths and leaves at water level, spreading via runner hyphae to the upper canopy under favorable microclimate conditions. Rice ShB initially causes oval to ellipsoid water-soaked lesions that eventually progress to a bleached, dry appearance surrounded by an irregular purple-brown border. Lesions may coalesce with one another eventually encompassing entire leaf sheaths or stems, which can result in stem lodging and reduced grain fill (Rush and Lee, 1983; Wu et al. 2012). Management of this disease is often difficult since the fungus may overwinter as sclerotia or dormant mycelia in crop debris and soil. Sclerotia are hardened masses of dormant mycelia that act as a resting body for the fungus. Sclerotia are formed on or around infected tissues and eventually drop to the ground where they can persist in the soil for up to two years and cause subsequent infection (Rush and Lee, 1983; Sumner, 1996). Sclerotia and dormant mycelia may also be spread early in the growing season with soil during field preparation or by movement with irrigation water during flooding (Wamishe et al. 2013).

Rice ShB poses a major constraint to both temperate and tropical rice production systems, affecting 15 to 20 million hectares and resulting in an annual grain yield loss of 6 million tons in

China alone (Bernardes de Assis et al. 2009). Sheath blight is also the most important rice disease in the southern United States and is present in almost all rice production fields in Arkansas, which is the top rice-producing state (Wamishe et al. 2013). Sheath blight severity in the southern U.S. is also exacerbated by crop rotations with soybean (an alternative host for the intraspecific group 1A of *Rhizoctonia solani*), high planting densities, excessive use of nitrogen fertilizers, and planting of long grain varieties which are more susceptible to ShB (Groth, 1991). Rice ShB losses of up to 50% have been observed when susceptible cultivars are used under favorable years for pathogen spread (Groth, 2008), but more commonly 10-30% yield loss is observed under proper management (Xue-Wen et al. 2008). California receives minimal losses due to disease pressure since the majority of production takes place in the Sacramento Valley where the lack of rain and high light intensity provides a non-conducive environment for disease spread. Losses to disease are much higher in the southern-producing states where the relative humidity is high, nights are warm, and frequent rain events occur (California Rice Commission, 2000).

4.2 Agricultural practices affecting disease incidence

Agricultural practices including excessive use of nitrogen fertilizers, high planting densities, and the use of dwarf, high tillering rice varieties can lead to increased rice sheath blight severity in fields (Yellareddygari et al. 2014). Savary et al. found that increased nitrogen fertilization resulted in more frequent host plant tissue contacts (leaf-to-leaf and leaf-to-sheath), which can facilitate the spread of ShB in the plant canopy, and an increased capacity of the plant canopy to retain moisture (Savary et al. 1995). High planting densities can lead to increased rice ShB infection as it results in more plant tissue contacts (Castilla et al. 1996). Dwarf, high tillering cultivars of rice are usually more conducive to ShB infection since the increased number of tillers and shortened plant canopy creates dense stands which would subsequently have reduced light infiltration and higher potentials to retain leaf moisture (Rush and Lee, 1983; Wu et al. 2014).

4.3 Rice sheath blight (ShB) control

4.3.1 Chemical control

Several fungicides are commonly used for the treatment of rice ShB. In the United States, the commercial fungicides benomyl, propiconazole, thiabendazole, iprodione, and copper plus sulfur have traditionally been used; however, benomyl and iprodione are no longer labeled for

sheath blight control (Groth et al. 1990; Groth 2008). More recently the strobilurin fungicide azoxystrobin has been labeled for the control of rice ShB in the US and has been shown to be more effective than other fungicides, however it is more expensive and can be ineffective the longer it is applied after the heading stage (Groth, 2008; Groth, 2005). Several broad spectrum fungicides are also utilized worldwide for the control of rice ShB including other triazoles such as hexaconazole and tebuconazole, as well as other fungicides belonging to other classes including carbendazim, validamycin, and clopyralid (Kumar et al. 2016).

4.3.2 Biological control using PGPR

The frequent use of pesticides in agriculture can have detrimental effects on non-target organisms (Pimental and Levitan, 1986) as well as the environment (Manuel et al. 2008). The use of biological control agents could offer a more sustainable and environmentally friendly approach for combating pest populations in agriculture. While biological control agents may include bacteria, fungi, or beneficial insects the focus of this review will be on PGPR.

Several PGPR strains have been used for the suppression of rice ShB, namely members from the genera *Bacillus* and *Pseudomonas*. Various *Pseudomonas* strains effectively reduced rice ShB severity in greenhouse and field conditions in one study (Nandakumar et al. 2001a) with greater reductions in disease observed with PGPR mixtures utilizing more than one strain. These PGPR mixtures also resulted in increased levels of plant growth promotion and reduced disease severity caused by *Rhizoctonia solani*. In another study, *Pseudomonas* strains PF1 and FP7 induced heightened levels of peroxidase activity in rice plants resulting in increased levels of protection against *Rhizoctonia solani* (Nandakumar et al. 2001b). Rice plants treated with the bacterial strains exhibited high levels of peroxidase activity 96 hours after pathogen inoculation compared to treatments without bacteria where peroxidase expression disappeared 72 hours after challenging with pathogen. A strain of *Bacillus megaterium* reduced disease severity caused by *R. solani* in both greenhouse and field conditions (Kanjanamaneesathian et al. 2007). In the greenhouse study, a pellet formulation of *B. megaterium* significantly reduced the percentage of tillers exhibiting symptoms of ShB infection compared to the control and was statistically the same as the fungicide iprodione. Similarly in field conditions, a water-soluble granular formulation of *B. megaterium* significantly reduced the percentage of tillers exhibiting symptoms of ShB infection similar to the fungicide treatment iprodione, as well as significantly reduced the lesion length caused by the ShB pathogen compared to the untreated control.

Chapter 2: Evaluation and selection of *Paenibacillus* strains for their potential as biocontrol agents of *Rhizoctonia solani*

1. Introduction

Rice is one of the most important crops worldwide, accounting for more than half of the calories consumed in some Asian diets and serving as a staple food crop for half of the world's population (Khush, 2005). Like other crops, rice productivity and yield is limited by the proliferation of plant pathogens. One such pathogen of extreme importance is the basidiomycete fungus, *Rhizoctonia solani*, which can cause yield losses of up to 50% in favorable years (Groth, 2008). Rice production is estimated to need to increase by 40% by 2030 to keep up with the growing world population (Khush, 2005), which is expected to reach 8.6 billion by that time (United Nations, 2017). Hence, the control of the pathogenic fungus *R. solani* could contribute to the increase in yield needed to sustain the growing population.

The use of biological control agents could offer one such solution that is more sustainable and with less detrimental effects to the environment than pesticides (Pimental and Levitan, 1986; Manuel et al. 2008). Plant growth-promoting rhizobacteria (PGPR) are a group of beneficial, plant-associated bacteria that exhibit a variety of mechanisms applicable to protect crop plants against pathogens (Bhattacharyya and Jha, 2012). These mechanisms can be broken down into direct protection through antagonism of the pathogenic organism by the secretion of antibiotics, lytic enzymes, or other antimicrobial compounds (antibiosis), and into indirect protection by a mechanism known as induced systemic resistance (ISR) which primes innate plant defenses to have a more efficient response to pathogen infection (Compant et al. 2005).

The bacterial genus *Paenibacillus* was chosen for evaluation as biocontrol agents in this research project because it has received less attention in comparison to other more commonly evaluated PGPR genera and because *Paenibacillus* species are known to produce a large and diverse array of antimicrobial compounds with activity against both fungi and bacteria (Beatty and Jensen, 2002; Jung et al. 2003; Choi et al. 2008; Tambadou et al. 2015; Cochrane and Vederas, 2016). This knowledge lead us to hypothesize that the genus *Paenibacillus* may harbor potent biocontrol agents of *Rhizoctonia solani*.

2. Materials and Methods

2.1 *In vitro* assessment of antagonism to *Rhizoctonia solani*

2.1.1 PGPR Strains, Pathogen, Media, and Culture

PGPR strains in the culture collection at Auburn University belonging to the genus *Paenibacillus* were screened for their potential as biocontrol agents of the fungal pathogen *Rhizoctonia solani*. Strains were previously identified using 16S rDNA sequencing. Prior to use, all strains were stored in tryptic soy broth amended with 30% glycerol at -80ºC. Each strain was then taken from cold storage, streaked onto TSA plates, and then incubated at 28ºC for 48 hrs and checked for purity. Bacterial suspensions to be used for *in vitro* assessment were quantified using the McFarland standards (McFarland, 1907) and checked using serial dilutions or by optical density (nm = 600) readings using a Genesys 10S UV-Vis Spectrophotometer. *R. solani* was stored on agar slants of potato dextrose agar (PDA) prior to use in assays. For use in the assays, the fungus was transferred to a fresh PDA plate, incubated at 25ºC, and allowed to grow until it reached the edge of the plate (3-4 days).

2.1.2 Preliminary Antibiosis Assay

Paenibacillus strains were screened through a preliminary antibiosis test to determine which strains exhibited antagonism towards *R. solani*. Any strain showing inhibition or suppression of the fungus was then tested in advanced antibiosis testing. Strains were screened using a modified antibiosis technique utilizing different agars following a previous method (Liu et al. 2017) with slight modifications. Antibiosis tests were carried out on PDA plates in which 4 holes were cut out equidistant from each other using a 10 mm cork borer. The agar from the cut out holes was then removed using a sterile scalpel and the hole was filled with hot TSA. The new plates containing 4 TSA discs within a PDA plate were then allowed to sit for 2 days to determine if there was any contamination. If no contamination was present, 5 µl of suspensions of each strain (adjusted to an approximate concentration of 10^7 cfu/ml) were pipetted on to the top of each TSA disc and incubated for 48 hrs at 28ºC. Three strains and one control using sterile water were screened per plate. PGPR strain MBI600 (*Bacillus velezensis*), known for its broad spectrum antagonism against plant pathogens, was used as a positive control in this screening. After incubation for 2 days, a single 5 mm plug of *R. solani* was taken from the edge of a growing culture and was transferred to the center of the antibiosis plate. The plates were then incubated at room temperature for 72 hrs before determining if the strains exhibited any antagonism to the

fungus. Any strains positive for antibiosis were carried on to other *in vitro* assays described below to characterize the antagonistic capability of strains to *Rhizoctonia solani*.

2.1.3 Advanced Antibiosis Assay

Plates were prepared in the same way as stated above except only 2 TSA discs were made in each plate. Each strain was pipetted as stated above onto TSA discs. Each disc was considered a separate replication, so 2 plates in total were used per strain. Bacterial strains were incubated, and the fungus was transferred as described above. After incubation, the zones of inhibition were measured in millimeters for each strain using a slightly modified method (Baysal et al. 2008). The zone of inhibition was defined as the area of clearing between the edge of the growing bacteria and inhibited mycelia (Baysal et al. 2008).

2.1.4 Inhibition of Sclerotia Germination Assay

All strains that were positive in the preliminary antibiosis assay were screened for their potential to inhibit the germination of sclerotia of *Rhizoctonia solani* following a previous method with slight modifications (Kazempour, 2004). Sclerotia were produced by transferring a 5 mm plug of actively growing mycelia to fresh PDA plates and were incubated under darkness for 10 days at 25ºC. After 10 days young, uniform sclerotia were collected from cultures of *R. solani,* were surface sterilized using a 2.5% hypochlorite solution for 2 minutes, and then were washed for 1 minute in sterile water. Four surface-sterilized sclerotia were then placed into broth cultures of overnight-grown bacteria (approximate concentration of 10^8 cfu/ml) and incubated for 24 hrs on a shaking incubator at 25ºC at 180 rpm. Sclerotia were then removed from broth cultures and placed onto PDA plates divided into 4 quadrants (1 sclerotium per quadrant) and incubated 48 hrs at 25ºC. After incubation the following formula was used to quantify percent inhibition where Control = growth of mycelia in the control treatment and Treatment = growth of mycelia in the treatment group.

$$\% \, Inhibition = \frac{100\,(Control - Treatment)}{Control}$$

Twenty-one strains were screened per experiment with four replications per treatment group. The experiment was repeated once, and results were pooled for statistical analysis.

2.1.5 Detached Leaf Assay

Strains were screened in an *in vivo* detached leaf assay to evaluate their potential to suppress the spread of *R. solani* on rice leaves. Rice plants were grown in sandy-loam field soil obtained

from E.V. Smith Research Center located in Shorter, Alabama in a greenhouse setting under fertilizer recommendations determined by a soil test for 60 days prior to use for this assay. After 60 days, rice leaves were harvested by cutting into 6-cm segments and were stored in plastic bags placed into a cooler. The second and third leaves from the bottom of the plant were used for this assay. The leaf tips and bottoms of leaves were avoided and only the middle of the leaves with uniform widths were used. Leaf pieces were then sterilized with a 1% hypochlorite solution for 1 minute and then washed with sterile water for 1 minute. Leaf pieces were then transferred to standard 15 cm petri dishes containing 1% water agar (1 leaf piece per plate) and the edges were carefully slid into the agar so that the leaves remained flat. Overnight-grown cultures of strains at a concentration of 10^8 cfu/ml were then spread on to leaves using a sterile cotton swab. Water was used in place of bacterial cultures for the negative control. A single sclerotium was then transferred to the center of each leaf piece. Sclerotia were produced as stated above by incubating *R. solani* for 10 days under darkness. Petri dishes containing the rice leaves were then placed into a growth chamber maintained at 25℃ on a 12-hr photoperiod for 7 days. At the end of incubation, leaves were then evaluated based upon the percentage of leaf area covered by lesions using the following disease scale:

0: 0%   1: 1-15%   2: 16-30%   3: 31-45%   4: 46-60   5: 61-75%   6: 76-90%   7: 91-100%

Twenty-one strains were screened per experiment with four replications per treatment group. The experiment was repeated once and results were pooled for statistical analysis.

2.2 Assessment of best inoculation technique for *Rhizoctonia solani*

A preliminary test was conducted in order to determine the best inoculation technique for *Rhizoctonia solani*, to optimize development of sheath blight disease in the greenhouse. Square, black pots measuring 10.5 cm across and 12.5 cm deep were used in this study. The bottom of the pots were wrapped with black landscape fabric and secured using clear packing tape to prevent soil from leaking out during flooded conditions. Pots were then filled with 1,300 g of fine sandy loam mineral field soil (80% sand, 10 % silt, and 10% clay) obtained from E.V. Smith Research Center located in Shorter, Alabama. Phosphorus applied as triple super phosphate (0-45-0) and potassium applied as muriate of potash (0-0-60) were amended into the soil at this stage following the recommendation of a soil test. Three seeds of the rice cultivar CL111 were sowed per pot to a depth of 2.5 cm and hand-watered to field capacity until the seedlings had reached fourth leaf stage. CL111 is an early maturing, dwarf, long grain rice that is susceptible to

rice sheath blight (ShB), and was developed at the LSU's AgCenter's H. Rouse Caffey Rice Research Station in Crowley, Louisiana, USA (Oard et al. 2014). At the fourth leaf stage, the strongest plant per pot was kept and the rest were thinned so that only one plant remained. Each pot was then placed into its own individual clear, round plastic container measuring 16.8 cm across and 11 cm deep. Nitrogen fertilizer was applied at this stage in the form of urea (44-0-0) following the recommendation of a soil test. Respective inoculation treatments were then applied and pots were maintained at a constant flood by keeping plastic containers full of water at all times.

Various methods of applying the pathogen were used in this experiment following a previous method with modifications (Park et al. 2008), including inoculations with a single sclerotium, single agar plug, and single mycelial ball. Plants inoculated with water served as an untreated control in this experiment. Sclerotia to be used for this experiment were produced as stated above in the "Inhibition of Sclerotia Germination Assay" section. For agar plug production, a 2 mm agar plug was aseptically transferred from the edge of an actively growing 3-4 day old culture of *Rhizoctonia* solani. Mycelial balls were produced by transferring a 5 mm plug of actively growing mycelia to a 250 ml Erlenmeyer flask containing 150 ml of full strength potato dextrose broth. Flasks were placed onto a shaking incubator at 150 rpm at 25ºC and were allowed to grow for 7 days. The resulting mycelial ball was then removed from the flasks and cut into sections. Sections were then briefly blotted on sterile filter paper and then weighed to 0.1 g. These 0.1 g mycelial balls were then used for inoculation.

Plants were treated by placing their respective inoculant at the base between two leaf sheaths. The inoculation site was then wrapped with aluminum foil for 5 days. After 5 days aluminum foil was removed and disease development was allowed to progress for an additional 10 days. At the end of 15 days post inoculation, disease development was assessed using the following formula based on the total height of all lesions present on a leaf sheath divided by the total height of the sheath and then multiplied by 100:

$$Lesion\ height\ (\%) = \frac{total\ height\ of\ all\ lesions}{sheath\ height} x\ 100$$

Up to 10 sheaths were measured per plant, and these values were averaged to represent a single plant. Each treatment contained 5 replications, and each experiment was arranged in a

randomized complete block design (RCBD). The experiment was repeated twice to validate results.

2.3 *In planta* assessment of biocontrol candidates in a greenhouse setting

Mycelial ball inoculation was chosen for disease bioassays based on the high amount of disease that it caused in the inoculation methods test. A single mycelial ball was used for inoculation of plants in the inoculation methods test, however two mycelial balls, each weighing 0.1 g, were used for inoculation per plant in this experiment to increase the amount of disease. Rice plants were planted and fertilized as stated above. After 4 weeks, plants were inoculated with *Paenibacillus* biocontrol candidates as a foliar spray at a concentration of 10^8 cfu/ml using a hand sprayer. Bacterial suspensions were made by suspending bacterial colonies grown on TSA plates in sterile water. Bacterial suspensions were then sprayed onto plants thoroughly covering all leaves and sheaths until just before runoff. Plants were inoculated as stated above 24 hrs after treatment with PGPR candidates and disease symptoms were allowed to develop for 15 days after inoculation before assessing with the total lesion height percentage formula. In addition to the lesion height formula, a disease rating scale was established. The disease rating scale was based on the percentage of sheath covered by lesion and was established as a ten point scale:
0: 0% **1:** 1-10% **2:** 11-20% **3:** 21-30% **4:** 31-40% **5:** 41-50% **6:** 51-60% **7:** 61-70%
8: 71-80% **9:** 81-90% **10:** 91-100%

2.4 Statistical Analysis

All data were analyzed by analysis of variance (ANOVA) to test for significance at the critical value ($P \leq 0.05$) using R statistical analysis software. Treatment means were then separated using Fisher's protected least significant difference (LSD) test using the "agricolae" package at $P \leq 0.05$ for *in vitro* experiments and the preliminary inoculation technique experiments. For disease bioassays in the greenhouse treatment means were separated using Dunnett's method at $P \leq 0.05$ with treatment groups being compared to the inoculated or un-inoculated disease control using the "multcomp" package.

3. Results

3.1 *In vitro* assessment of antagonism to *Rhizoctonia solani*

3.1.1 Antibiosis Screenings

In the preliminary antibiosis screening, 335 *Paenibacillus* strains were screened for antibiosis against the fungal rice sheath blight pathogen *Rhizoctonia solani* (Table 1). Twenty-one strains showed antagonism to *Rhizoctonia solani* and were carried on to advanced antibiosis testing where their zones of inhibition were measured (Table 2). The strains positive for antibiosis interestingly fell into one of 3 species: *Paenibacillus peoriae*, *Paenibacillus jamilae,* or *Paenibacillus polymyxa*. In advanced antibiosis testing, strains JJ-227 (*P. peoriae*) and JJ-1720 (*P. peoriae*) exhibited statistically larger inhibition zones, 11.37 mm and 11.25 mm respectively, than the positive control strain MBI600, while strains JJ-1824, JJ-16, JJ-195, JJ-333, JJ-845, and JJ-165 had significantly smaller zones of inhibition (9.25 mm, 9.00 mm, 8.75 mm, 8.25 mm, 8.00 mm, and 7.37 mm respectively) compared to the positive control strain MBI600 (Table 3). Regardless of significance compared to the positive control strain all strains exhibiting antibiosis were carried on to the sclerotia germination and detached leaf assays.

3.1.2 Effect of biocontrol candidates on sclerotia germination

All *Paenibacillus* strains completely inhibited the germination of sclerotia from *Rhizoctonia solani*, with the exception of JJ-226, which inhibited mycelial growth by 60.01%, at a concentration of 10^8 cfu/ml when co-cultured together for 24 hrs and then plated onto PDA (Table 3).

3.1.3 Effect of *Paenibacillus* strains on disease severity in a detached leaf assay

Several strains significantly reduced the disease severity caused by *Rhizoctonia solani*. Compared to the disease control, strains JJ-16, JJ-165, JJ-195, JJ-228, JJ-333, JJ-845, JJ-1580, JJ-1710, and JJ-1824 had significantly lower disease ratings (Table 3). All of the strains that performed well in the detached leaf assay were either strains of *P. peoriae* or *P. jamilae*. Strain JJ-1710 (*Paenibacillus jamilae*) was the top performing strain in the detached leaf assay.

3.2 Assessment of best inoculation technique for *Rhizoctonia solani*

Three methods of inoculation were evaluated for their capacity to cause significant disease on rice, including single agar plug (2 mm) inoculation, sclerotium inoculation, or mycelial ball inoculation. All inoculation methods resulted in disease with higher lesion height percentages. The non-inoculated control pots did not display any disease symptoms. Agar plug inoculation

resulted in the least amount of disease with a mean lesion length of 7.6% (Table 4). Sclerotium and mycelial ball inoculation resulted in significantly more disease with mean lesion lengths of 15.5% and 17.9% respectively (Table 4). Sclerotium and mycelial ball inoculation were not statistically different from each other; however, the mycelial ball inoculation trended higher in disease severity and as such it was chosen as the inoculation method for the greenhouse disease bioassays.

3.3 *In planta* assessment of biocontrol candidates in a greenhouse setting

Nine strains in total were evaluated for their antagonistic effects on *R. solani* in greenhouse disease bioassays. Six strains (JJ-195, JJ-228, JJ-333, JJ-1580, JJ-1710, and JJ-1824) resulted in significantly reduced disease severity compared to the disease control (19.03%) with lesion length means of 14.5%, 14.2%, 14.1%, 13.9%, 12.4%, and 11.4% respectively (Table 4). JJ-1710 and JJ-1824 ($P < 0.0001$) were top performers in the greenhouse assays. Additionally, four strains (JJ-195, JJ-1580, JJ-1710, and JJ-1824) had significantly reduced disease ratings compared to the disease control (1.66) with mean disease rating values of 1.31, 1.27, 1.28, and 1.17 respectively (Table 5).

4. Discussion

Dual culture antibiosis assays are a common preliminary step for evaluating the potential of PGPR to suppress the growth of a pathogen (Schoina et al. 2011; Niu et al. 2013; Liu et al. 2017). Sclerotia germination and detached leaf assays are also common for assessing the antagonistic potential of PGPR strains to *Rhizoctonia solani* causing rice sheath blight (Kazempour, 2004; Shrestha et al. 2016).

In summary of this study, 21 of the 335 evaluated strains of bacteria belonging to the genus *Paenibacillus* showed antagonistic activity to *Rhizoctonia solani* in preliminary antibiosis assays. Interestingly, of the 56 species of *Paenibacillus* evaluated in this study, only multiple strains of 3 species (*P. peoriae, P. jamilae,* and *P. polymyxa*) showed antagonistic activity to *R. solani*. In advanced antibiosis testing, two strains identifying as *P. peoriae* strains exhibited the largest inhibition zones to *R. solani.* However, caution should be exercised when using inhibition zone size *in vitro* as a cutoff for biocontrol candidate strains. For example, 6 of the 9 strains that significantly lowered disease severity in the detached leaf assay produced the smallest inhibition zones in advanced antibiosis testing. Among the six strains producing the smallest inhibition

zones, strain JJ-1824 was one of the two top performers in the greenhouse disease assay, reducing mean lesion height by 39.9%.

Six out of the nine strains that were evaluated in greenhouse trials had significantly lower total lesion height percentages, and four of these strains (JJ-195, JJ-1580, JJ-1710, JJ-1824) also had significantly lower disease ratings compared to the disease control. JJ-195, JJ-1580, and JJ-1710 were strains of *P. peoriae* and JJ-1824 was a strain of *P. jamilae*. The results of these experiments demonstrate that strains of *Paenibacillus peoriae* and *Paenibacillus jamilae* can significantly reduce the amount of disease caused by the fungus *Rhizoctonia solani* in rice. The capacity of *P. polymyxa* strains to inhibit fungal pathogens is well-documented (Beatty and Jensen, 2002; Li et al. 2007; Choi et al. 2008); however, strains of *P. polymyxa* were not carried to greenhouse trials in this experiment since they did not significantly reduce the amount of disease caused by *R. solani* in the detached leaf assay. Reports of fungal suppression by strains of *P. peoriae* are very limited, and all of them seem to have been done only *in vitro* (Von der Weid et al. 2003; Lorentz et al. 2006; Xu and Kim, 2014). Von der Weid et al. was the first to report antimicrobial production by *P. peoriae* (Von der Weid et al. 2003), but there do not appear to be many cases exploring the biocontrol capabilities of this species. To our knowledge this is the first published report of plant disease biocontrol by a strain of *P. jamilae*.

Table 1. All *Paenibacillus* strains screened through the preliminary antibiosis assay

Strain ID	Genus	Species	16S Match	Strain ID	Genus	Species	16S Match
JJ-6	*Paenibacillus*	*xylanexedens*	99.39%	JJ-121	*Paenibacillus*	*lautus*	99.23%
JJ-7	*Paenibacillus*	*typhae*	98.70%	JJ-148	*Paenibacillus*	*chondroitinus*	97.94%
JJ-15	*Paenibacillus*	*amylolyticus*	99.59%	JJ-159	*Paenibacillus*	*lautus*	99.23%
JJ-16	*Paenibacillus*	*peoriae*	99.59%	JJ-160	*Paenibacillus*	*terrigena*	98.77%
JJ-18	*Paenibacillus*	*alginolyticus*	99.22%	JJ-165	*Paenibacillus*	*jamilae*	99.86%
JJ-21	*Paenibacillus*	*peoriae*	99.66%	JJ-174	*Paenibacillus*	*amylolyticus*	99.25%
JJ-29	*Paenibacillus*	*illinoisensis*	99.65%	JJ-180	*Paenibacillus*	*chondroitinus*	98.01%
JJ-33	*Paenibacillus*	*lautus*	99.37%	JJ-190	*Paenibacillus*	*jilunlii*	99.79%
JJ-39	*Paenibacillus*	*terrigena*	99.25%	JJ-193	*Paenibacillus*	*terrigena*	99.52%
JJ-42	*Paenibacillus*	*pectinilyticus*	98.78%	JJ-194	*Paenibacillus*	*amylolyticus*	99.32%
JJ-47	*Paenibacillus*	*cineris*	100.00%	JJ-195	*Paenibacillus*	*peoriae*	99.72%
JJ-49	*Paenibacillus*	*lautus*	99.16%	JJ-216	*Paenibacillus*	*pocheonensis*	99.04%
JJ-55	*Paenibacillus*	*borealis*	98.29%	JJ-223	*Paenibacillus*	*xylanilyticus*	98.37%
JJ-56	*Paenibacillus*	*amylolyticus*	99.45%	JJ-226	*Paenibacillus*	*peoriae*	99.79%
JJ-59	*Paenibacillus*	*chondroitinus*	97.87%	JJ-227	*Paenibacillus*	*peoriae*	99.79%
JJ-60	*Paenibacillus*	*odorifer*	98.32%	JJ-228	*Paenibacillus*	*peoriae*	99.79%
JJ-64	*Paenibacillus*	*borealis*	97.83%	JJ-231	*Paenibacillus*	*illinoisensis*	99.86%
JJ-72	*Paenibacillus*	*amylolyticus*	99.80%	JJ-232	*Paenibacillus*	*dongdonensis*	99.19%
JJ-73	*Paenibacillus*	*terrigena*	99.04%	JJ-235	*Paenibacillus*	*amylolyticus*	99.66%
JJ-76	*Paenibacillus*	*chondroitinus*	98.92%	JJ-237	*Paenibacillus*	*amylolyticus*	99.66%
JJ-77	*Paenibacillus*	*chondroitinus*	98.92%	JJ-246	*Paenibacillus*	*oenotherae*	98.63%
JJ-90	*Paenibacillus*	*typhae*	98.70%	JJ-249	*Paenibacillus*	*thermophilus*	99.72%
JJ-92	*Paenibacillus*	*lautus*	99.30%	JJ-253	*Paenibacillus*	*pectinilyticus*	98.78%
JJ-93	*Paenibacillus*	*chondroitinus*	98.01%	JJ-268	*Paenibacillus*	*borealis*	98.10%
JJ-95	*Paenibacillus*	*chondroitinus*	98.98%	JJ-270	*Paenibacillus*	*borealis*	97.97%
JJ-99	*Paenibacillus*	*chondroitinus*	98.01%	JJ-272	*Paenibacillus*	*borealis*	98.72%
JJ-100	*Paenibacillus*	*amylolyticus*	97.96%	JJ-277	*Paenibacillus*	*chondroitinus*	97.87%
JJ-102	*Paenibacillus*	*xylanexedens*	99.39%	JJ-278	*Paenibacillus*	*terrigena*	99.66%
JJ-106	*Paenibacillus*	*marinesediminis*	99.59%	JJ-287	*Paenibacillus*	*agaridevorans*	97.41%
JJ-299	*Paenibacillus*	*oenotherae*	98.29%	JJ-413	*Paenibacillus*	*'taohuashanense'*	98.80%
JJ-305	*Paenibacillus*	*illinoisensis*	99.72%	JJ-415	*Paenibacillus*	*catalpae*	99.59%
JJ-310	*Paenibacillus*	*endophyticus*	99.52%	JJ-447	*Paenibacillus*	*ehimensis*	96.56%

Table 1. All *Paenibacillus* strains screened through the preliminary antibiosis assay

Strain ID	Genus	Species	16S Match	Strain ID	Genus	Species	16S Match
JJ-311	*Paenibacillus*	*amylolyticus*	98.64%	JJ-450	*Paenibacillus*	*pectinilyticus*	99.20%
JJ-317	*Paenibacillus*	*'taohuashanense'*	99.86%	JJ-460	*Paenibacillus*	*pectinilyticus*	99.78%
JJ-318	*Paenibacillus*	*illinoisensis*	99.79%	JJ-467	*Paenibacillus*	*pectinilyticus*	98.85%
JJ-324	*Paenibacillus*	*lautus*	99.37%	JJ-471	*Paenibacillus*	*sabinae*	99.46%
JJ-329	*Paenibacillus*	*'yonginensis'*	97.47%	JJ-483	*Paenibacillus*	*panacisoli*	99.65%
JJ-331	*Paenibacillus*	*'taohuashanense'*	98.66%	JJ-497	*Paenibacillus*	*amylolyticus*	99.59%
JJ-332	*Paenibacillus*	*catalpae*	99.59%	JJ-514	*Paenibacillus*	*dongdonensis*	99.12%
JJ-333	*Paenibacillus*	*jamilae*	99.86%	JJ-526	*Paenibacillus*	*lautus*	99.21%
JJ-337	*Paenibacillus*	*illinoisensis*	99.79%	JJ-534	*Paenibacillus*	*barcinonensis*	99.11%
JJ-338	*Paenibacillus*	*lautus*	99.37%	JJ-539	*Paenibacillus*	*amylolyticus*	98.78%
JJ-340	*Paenibacillus*	*thiaminolyticus*	99.79%	JJ-541	*Paenibacillus*	*panacisoli*	99.72%
JJ-342	*Paenibacillus*	*terrigena*	99.52%	JJ-556	*Paenibacillus*	*massiliensis*	99.66%
JJ-352	*Paenibacillus*	*chondroitinus*	98.30%	JJ-591	*Paenibacillus*	*graminis*	99.79%
JJ-355	*Paenibacillus*	*illinoisensis*	99.72%	JJ-597	*Paenibacillus*	*anaericanus*	98.65%
JJ-364	*Paenibacillus*	*urinalis*	99.35%	JJ-602	*Paenibacillus*	*alvei*	99.19%
JJ-365	*Paenibacillus*	*odorifer*	99.51%	JJ-606	*Paenibacillus*	*alvei*	99.19%
JJ-372	*Paenibacillus*	*chondroitinus*	99.00%	JJ-616	*Paenibacillus*	*amylolyticus*	99.66%
JJ-380	*Paenibacillus*	*rigui*	98.18%	JJ-618	*Paenibacillus*	*lentus*	98.37%
JJ-384	*Paenibacillus*	*chondroitinus*	97.94%	JJ-624	*Paenibacillus*	*alkaliterrae*	99.33%
JJ-385	*Paenibacillus*	*graminis*	99.38%	JJ-626	*Paenibacillus*	*massiliensis*	99.72%
JJ-391	*Paenibacillus*	*illinoisensis*	99.65%	JJ-631	*Paenibacillus*	*lautus*	99.09%
JJ-393	*Paenibacillus*	*terrigena*	99.17%	JJ-632	*Paenibacillus*	*illinoisensis*	99.79%
JJ-402	*Paenibacillus*	*terrigena*	99.79%	JJ-633	*Paenibacillus*	*lautus*	99.09%
JJ-407	*Paenibacillus*	*oenotherae*	98.56%	JJ-662	*Paenibacillus*	*'taohuashanense'*	98.73%
JJ-408	*Paenibacillus*	*terrigena*	99.04%	JJ-666	*Paenibacillus*	*'humi'*	99.79%
JJ-410	*Paenibacillus*	*borealis*	97.84%	JJ-667	*Paenibacillus*	*chondroitinus*	97.94%
JJ-411	*Paenibacillus*	*chondroitinus*	97.94%	JJ-676	*Paenibacillus*	*'taohuashanense'*	98.59%
JJ-412	*Paenibacillus*	*lautus*	99.23%	JJ-690	*Paenibacillus*	*lautus*	99.58%
JJ-702	*Paenibacillus*	*tundrae*	99.46%	JJ-1004	*Paenibacillus*	*barcinonensis*	98.69%
JJ-717	*Paenibacillus*	*'taohuashanense'*	98.87%	JJ-1037	*Paenibacillus*	*amylolyticus*	98.98%
JJ-718	*Paenibacillus*	*'taohuashanense'*	98.73%	JJ-1044	*Paenibacillus*	*dongdonensis*	99.39%
JJ-754	*Paenibacillus*	*panacisoli*	99.59%	JJ-1057	*Paenibacillus*	*amylolyticus*	99.80%

Table 1. All *Paenibacillus* strains screened through the preliminary antibiosis assay

Strain ID	Genus	Species	16S Match	Strain ID	Genus	Species	16S Match
JJ-764	*Paenibacillus*	*castaneae*	97.93%	JJ-1059	*Paenibacillus*	*lautus*	99.23%
JJ-773	*Paenibacillus*	*amylolyticus*	99.59%	JJ-1064	*Paenibacillus*	*lautus*	99.09%
JJ-778	*Paenibacillus*	*amylolyticus*	99.80%	JJ-1069	*Paenibacillus*	*barcinonensis*	98.69%
JJ-779	*Paenibacillus*	*lactis*	99.51%	JJ-1074	*Paenibacillus*	*amylolyticus*	99.86%
JJ-782	*Paenibacillus*	*amylolyticus*	99.73%	JJ-1099	*Paenibacillus*	*odorifer*	98.58%
JJ-788	*Paenibacillus*	*amylolyticus*	99.52%	JJ-1103	*Paenibacillus*	*xylanilyticus*	100.00%
JJ-794	*Paenibacillus*	*lautus*	99.44%	JJ-1112	*Paenibacillus*	*susongensis*	99.17%
JJ-796	*Paenibacillus*	*'taohuashanense'*	94.72%	JJ-1113	*Paenibacillus*	*alvei*	99.12%
JJ-798	*Paenibacillus*	*'taohuashanense'*	98.87%	JJ-1115	*Paenibacillus*	*amylolyticus*	99.59%
JJ-799	*Paenibacillus*	*'taohuashanense'*	98.73%	JJ-1140	*Paenibacillus*	*illinoisensis*	99.79%
JJ-816	*Paenibacillus*	*barcinonensis*	99.11%	JJ-1181	*Paenibacillus*	*terrigena*	99.17%
JJ-820	*Paenibacillus*	*tundrae*	99.59%	JJ-1187	*Paenibacillus*	*illinoisensis*	99.79%
JJ-824	*Paenibacillus*	*illinoisensis*	99.86%	JJ-1275	*Paenibacillus*	*doosanensis*	98.56%
JJ-831	*Paenibacillus*	*borealis*	98.77%	JJ-1277	*Paenibacillus*	*dongdonensis*	99.12%
JJ-832	*Paenibacillus*	*borealis*	98.77%	JJ-1279	*Paenibacillus*	*thiaminolyticus*	99.93%
JJ-845	*Paenibacillus*	*jamilae*	99.52%	JJ-1282	*Paenibacillus*	*graminis*	99.93%
JJ-870	*Paenibacillus*	*castaneae*	99.32%	JJ-1283	*Paenibacillus*	*pocheonensis*	98.97%
JJ-882	*Paenibacillus*	*lautus*	99.22%	JJ-1311	*Paenibacillus*	*amylolyticus*	99.58%
JJ-890	*Paenibacillus*	*contaminans*	99.93%	JJ-1319	*Paenibacillus*	*barcinonensis*	99.17%
JJ-918	*Paenibacillus*	*amylolyticus*	99.86%	JJ-1323	*Paenibacillus*	*susongensis*	99.17%
JJ-955	*Paenibacillus*	*jilunlii*	98.04%	JJ-1343	*Paenibacillus*	*barcinonensis*	99.29%
JJ-960	*Paenibacillus*	*aestuarii*	95.04%	JJ-1371	*Paenibacillus*	*catalpae*	99.66%
JJ-961	*Paenibacillus*	*marinisediminis*	99.52%	JJ-1386	*Paenibacillus*	*barcinonensis*	99.11%
JJ-963	*Paenibacillus*	*borealis*	98.34%	JJ-1401	*Paenibacillus*	*susongensis*	99.16%
JJ-964	*Paenibacillus*	*lautus*	99.44%	JJ-1402	*Paenibacillus*	*susongensis*	99.17%
JJ-985	*Paenibacillus*	*castaneae*	99.39%	JJ-1405	*Paenibacillus*	*susongensis*	99.17%
JJ-1000	*Paenibacillus*	*xylanexedens*	99.86%	JJ-1410	*Paenibacillus*	*odorifer*	98.78%
JJ-1415	*Paenibacillus*	*selenitireducens*	99.23%	JJ-1570	*Paenibacillus*	*susongensis*	99.17%
JJ-1425	*Paenibacillus*	*granivorans*	96.71%	JJ-1574	*Paenibacillus*	*contaminans*	99.79%
JJ-1461	*Paenibacillus*	*barcinonensis*	99.24%	JJ-1580	*Paenibacillus*	*peoriae*	99.72%
JJ-1465	*Paenibacillus*	*amylolyticus*	98.71%	JJ-1582	*Paenibacillus*	*peoriae*	99.65%
JJ-1470	*Paenibacillus*	*amylolyticus*	99.45%	JJ-1586	*Paenibacillus*	*amylolyticus*	98.77%

Table 1. All *Paenibacillus* strains screened through the preliminary antibiosis assay

Strain ID	Genus	Species	16S Match	Strain ID	Genus	Species	16S Match
JJ-1471	*Paenibacillus*	*barcinonensis*	99.17%	JJ-1587	*Paenibacillus*	*illinoisensis*	99.72%
JJ-1475	*Paenibacillus*	*jilunlii*	97.99%	JJ-1588	*Paenibacillus*	*amylolyticus*	99.52%
JJ-1476	*Paenibacillus*	*massiliensis*	99.86%	JJ-1591	*Paenibacillus*	*taichungensis*	99.73%
JJ-1477	*Paenibacillus*	*typhae*	97.47%	JJ-1595	*Paenibacillus*	*odorifer*	98.48%
JJ-1481	*Paenibacillus*	*amylolyticus*	98.91%	JJ-1596	*Paenibacillus*	*barcinonensis*	99.17%
JJ-1485	*Paenibacillus*	*massiliensis*		JJ-1598	*Paenibacillus*	*odorifer*	99.80%
JJ-1486	*Paenibacillus*	*amylolyticus*	98.84%	JJ-1601	*Paenibacillus*	*illinoisensis*	99.79%
JJ-1487	*Paenibacillus*	*pocheonensis*	98.97%	JJ-1602	*Paenibacillus*	*typhae*	98.18%
JJ-1491	*Paenibacillus*	*susongensis*	99.22%	JJ-1603	*Paenibacillus*	*peoriae*	99.86%
JJ-1501	*Paenibacillus*	*lautus*	99.37%	JJ-1604	*Paenibacillus*	*polymyxa*	100.00%
JJ-1509	*Paenibacillus*	*barcinonensis*	99.24%	JJ-1608	*Paenibacillus*	*typhae*	99.52%
JJ-1516	*Paenibacillus*	*susongensis*	99.24%	JJ-1611	*Paenibacillus*	*typhae*	97.08%
JJ-1520	*Paenibacillus*	*jilunlii*	97.99%	JJ-1612	*Paenibacillus*	*barcinonensis*	99.24%
JJ-1522	*Paenibacillus*	*barcinonensis*	99.31%	JJ-1614	*Paenibacillus*	*peoriae*	99.65%
JJ-1524	*Paenibacillus*	*borealis*	98.34%	JJ-1615	*Paenibacillus*	*typhae*	99.45%
JJ-1525	*Paenibacillus*	*odorifer*	99.93%	JJ-1620	*Paenibacillus*	*massiliensis*	99.86%
JJ-1526	*Paenibacillus*	*jilunlii*	97.99%	JJ-1623	*Paenibacillus*	*dongdonensis*	99.12%
JJ-1529	*Paenibacillus*	*amylolyticus*	98.91%	JJ-1631	*Paenibacillus*	*dongdonensis*	99.27%
JJ-1531	*Paenibacillus*	*jilunlii*	97.85%	JJ-1636	*Paenibacillus*	*dongdonensis*	99.49%
JJ-1544	*Paenibacillus*	*barcinonensis*	99.17%	JJ-1638	*Paenibacillus*	*peoriae*	99.72%
JJ-1546	*Paenibacillus*	*jilunlii*	97.97%	JJ-1639	*Paenibacillus*	*amylolyticus*	98.84%
JJ-1549	*Paenibacillus*	*massiliensis*	99.73%	JJ-1640	*Paenibacillus*	*peoriae*	99.72%
JJ-1553	*Paenibacillus*	*amylolyticus*	98.84%	JJ-1642	*Paenibacillus*	*peoriae*	99.72%
JJ-1555	*Paenibacillus*	*barcinonensis*	99.31%	JJ-1647	*Paenibacillus*	*massiliensis*	99.86%
JJ-1563	*Paenibacillus*	*terrigena*	99.65%	JJ-1648	*Paenibacillus*	*turicensis*	97.36%
JJ-1564	*Paenibacillus*	*amylolyticus*	98.56%	JJ-1649	*Paenibacillus*	*illinoisensis*	99.79%
JJ-1650	*Paenibacillus*	*jamilae*	100.00%	JJ-1725	*Paenibacillus*	*amylolyticus*	99.66%
JJ-1652	*Paenibacillus*	*peoriae*	99.72%	JJ-1726	*Paenibacillus*	*dongdonensis*	99.27%
JJ-1653	*Paenibacillus*	*amylolyticus*	98.84%	JJ-1729	*Paenibacillus*	*polymyxa*	100.00%
JJ-1655	*Paenibacillus*	*typhae*	97.60%	JJ-1730	*Paenibacillus*	*typhae*	99.52%
JJ-1667	*Paenibacillus*	*odorifer*	99.86%	JJ-1731	*Paenibacillus*	*jilunlii*	97.90%
JJ-1669	*Paenibacillus*	*odorifer*	98.58%	JJ-1732	*Paenibacillus*	*typhae*	99.73%

Table 1. All *Paenibacillus* strains screened through the preliminary antibiosis assay

Strain ID	Genus	Species	16S Match	Strain ID	Genus	Species	16S Match
JJ-1671	*Paenibacillus*	*typhae*	99.45%	JJ-1734	*Paenibacillus*	*typhae*	99.16%
JJ-1672	*Paenibacillus*	*typhae*	99.45%	JJ-1736	*Paenibacillus*	*typhae*	99.59%
JJ-1677	*Paenibacillus*	*massiliensis*	99.86%	JJ-1739	*Paenibacillus*	*dongdonensis*	99.19%
JJ-1678	*Paenibacillus*	*amylolyticus*	98.84%	JJ-1742	*Paenibacillus*	*amylolyticus*	98.84%
JJ-1679	*Paenibacillus*	*amylolyticus*	98.77%	JJ-1743	*Paenibacillus*	*polymyxa*	99.93%
JJ-1680	*Paenibacillus*	*massiliensis*	99.86%	JJ-1744	*Paenibacillus*	*typhae*	99.43%
JJ-1683	*Paenibacillus*	*jamilae*	99.73%	JJ-1747	*Paenibacillus*	*polymyxa*	99.65%
JJ-1684	*Paenibacillus*	*amylolyticus*	98.23%	JJ-1748	*Paenibacillus*	*typhae*	99.59%
JJ-1686	*Paenibacillus*	*jamilae*	99.73%	JJ-1749	*Paenibacillus*	*peoriae*	99.64%
JJ-1688	*Paenibacillus*	*massiliensis*	99.86%	JJ-1755	*Paenibacillus*	*graminis*	100.00%
JJ-1692	*Paenibacillus*	*typhae*	99.59%	JJ-1759	*Paenibacillus*	*catalpae*	99.66%
JJ-1693	*Paenibacillus*	*amylolyticus*	99.52%	JJ-1761	*Paenibacillus*	*anaericanus/selenii*	98.64%
JJ-1696	*Paenibacillus*	*typhae*	97.33%	JJ-1762	*Paenibacillus*	*alginolyticus*	99.29%
JJ-1701	*Paenibacillus*	*typhae*	99.66%	JJ-1765	*Paenibacillus*	*anaericanus/selenii*	98.64%
JJ-1703	*Paenibacillus*	*typhae*	99.59%	JJ-1774	*Paenibacillus*	*susongensis*	99.21%
JJ-1705	*Paenibacillus*	*typhae*	99.66%	JJ-1781	*Paenibacillus*	*barcinonensis*	99.03%
JJ-1706	*Paenibacillus*	*peoriae*	99.72%	JJ-1782	*Paenibacillus*	*amylolyticus*	98.97%
JJ-1709	*Paenibacillus*	*massiliensis*	99.93%	JJ-1783	*Paenibacillus*	*sonchi*	98.64%
JJ-1710	*Paenibacillus*	*peoriae*	99.72%	JJ-1784	*Paenibacillus*	*taiwanensis*	99.66%
JJ-1713	*Paenibacillus*	*typhae*	99.52%	JJ-1789	*Paenibacillus*	*dongdonensis*	99.27%
JJ-1715	*Paenibacillus*	*peoriae*	99.72%	JJ-1790	*Paenibacillus*	*massiliensis*	99.80%
JJ-1720	*Paenibacillus*	*peoriae*	99.72%	JJ-1791	*Paenibacillus*	*typhae*	99.66%
JJ-1722	*Paenibacillus*	*peoriae*	99.72%	JJ-1792	*Paenibacillus*	*taichungensis*	99.73%
JJ-1723	*Paenibacillus*	*dongdonensis*	99.27%	JJ-1793	*Paenibacillus*	*typhae*	99.45%
JJ-1724	*Paenibacillus*	*peoriae*	99.72%	JJ-1794	*Paenibacillus*	*turicensis*	98.65%
JJ-1795	*Paenibacillus*	*dongdonensis*	99.27%	JJ-1831	*Paenibacillus*	*typhae*	97.33%
JJ-1796	*Paenibacillus*	*amylolyticus*	98.63%	JJ-1832	*Paenibacillus*	*typhae*	99.52%
JJ-1803	*Paenibacillus*	*amylolyticus*	98.70%	JJ-1834	*Paenibacillus*	*amylolyticus*	98.77%
JJ-1807	*Paenibacillus*	*barcinonensis*	99.24%	JJ-1835	*Paenibacillus*	*typhae*	99.45%
JJ-1808	*Paenibacillus*	*susongensis*	99.17%	JJ-1842	*Paenibacillus*	*jilunlii*	97.92%
JJ-1810	*Paenibacillus*	*susongensis*	99.24%	JJ-1844	*Paenibacillus*	*amylolyticus*	98.77%
JJ-1813	*Paenibacillus*	*typhae*	99.59%	JJ-1851	*Paenibacillus*	*doosanensis*	98.41%

Table 1. All *Paenibacillus* strains screened through the preliminary antibiosis assay

Strain ID	Genus	Species	16S Match	Strain ID	Genus	Species	16S Match
JJ-1816	*Paenibacillus*	*jilunlii*	97.84%	JJ-1852	*Paenibacillus*	*jilunlii*	97.72%
JJ-1817	*Paenibacillus*	*jilunlii*	97.85%	JJ-1853	*Paenibacillus*	*jilunlii*	98.04%
JJ-1818	*Paenibacillus*	*anaericanus*	98.64%	JJ-1860	*Paenibacillus*	*odorifer*	98.18%
JJ-1819	*Paenibacillus*	*contaminans*	99.93%	JJ-1866	*Paenibacillus*	*validus*	98.25%
JJ-1821	*Paenibacillus*	*massiliensis*	99.73%	JJ-1883	*Paenibacillus*	*jilunlii*	97.84%
JJ-1823	*Paenibacillus*	*polymyxa*	100.00%	AP-66	*Paenibacillus*	*barcinonensis*	99.50%
JJ-1824	*Paenibacillus*	*jamilae*	99.86%	AP-115	*Paenibacillus*	*amylolyticus*	100.00%
JJ-1826	*Paenibacillus*	*typhae*	97.41%	AP-294	*Paenibacillus*	*peoriae*	99.39%
JJ-1830	*Paenibacillus*	*odorifer*	99.93%				

Table 2. Summary of positive Paenibacillus strains in preliminary antibiosis screening

Strain ID	Species	Match (%)[a]	Strain ID	Species	Match (%)[a]
JJ16	*P. peoriae*	99.59	JJ1642	*P. peoriae*	99.72
JJ165	*P. jamilae*	99.86	JJ1706	*P. peoriae*	99.72
JJ195	*P. peoriae*	99.72	JJ1710	*P. peoriae*	99.72
JJ226	*P. peoriae*	99.79	JJ1720	*P. peoriae*	99.72
JJ227	*P. peoriae*	99.79	JJ1724	*P. peoriae*	99.72
JJ228	*P. peoriae*	99.79	JJ1743	*P. polymyxa*	99.93
JJ333	*P. jamilae*	99.86	JJ1747	*P. polymyxa*	99.65
JJ845	*P. jamilae*	99.52	JJ1749	*P. peoriae*	99.64
JJ1580	*P. peoriae*	99.72	JJ1823	*P. polymyxa*	100.00
JJ1614	*P. peoriae*	99.65	JJ1824	*P. jamilae*	99.86
JJ1640	*P. peoriae*	99.72			

[a] Percent match to species using 16S rRNA sequencing.

Table 3. Summary of *in vitro* antagonistic activity to *Rhizoctonia solani*

Strain ID	Species	Inhibition zone (mm)[a]	Germination inhibition (%)[b]	Disease severity rating[c]
JJ16	*Paenibacillus peoriae*	11.37 ghi	100 a	2.50 cde
JJ165	*Paenibacillus jamilae*	7.37 k	100 a	2.13 de
JJ195	*Paenibacillus peoriae*	8.75 hij	100 a	2.25 cde
JJ226	*Paenibacillus peoriae*	10.12 cdef	60.1 b	2.75 abcde
JJ227	*Paenibacillus peoriae*	11.37 a	100 a	3.00 abcde
JJ228	*Paenibacillus peoriae*	11.12 ab	100 a	2.63 bcde
JJ333	*Paenibacillus jamilae*	8.25 ijk	100 a	2.13 de
JJ845	*Paenibacillus jamilae*	8.00 jk	100 a	2.25 cde
JJ1580	*Paenibacillus peoriae*	10.12 cdef	100 a	2.38 cde
JJ1614	*Paenibacillus peoriae*	10.25 bcde	100 a	3.13 abcd
JJ1640	*Paenibacillus peoriae*	9.62 efgh	100 a	3.38 abc
JJ1642	*Paenibacillus peoriae*	11.00 abc	100 a	3.88 a
JJ1706	*Paenibacillus peoriae*	10.62 abcd	100 a	3.25 abcd
JJ1710	*Paenibacillus peoriae*	9.75 defg	100 a	1.88 e
JJ1720	*Paenibacillus peoriae*	11.25 a	100 a	2.75 abcde
JJ1724	*Paenibacillus peoriae*	10.62 abcd	100 a	2.88 abcde
JJ1743	*Paenibacillus polymyxa*	9.75 defg	100 a	3.13 abcd
JJ1747	*Paenibacillus polymyxa*	10.75 abc	100 a	3.13 abcd
JJ1749	*Paenibacillus peoriae*	10.50 abcde	100 a	3.75 ab
JJ1823	*Paenibacillus polymyxa*	9.75 defg	100 a	2.75 abcde
JJ1824	*Paenibacillus jamilae*	9.25 fgh	100 a	2.25 cde
MBI600	*Bacillus velezensis*	10.25 bcde	100 a	3.25 abcd
Control		0 i	0 c	3.87 a
$LSD_{0.05}$		0.97	3.17	1.14

[a] Mean values for inhibition zone in antibiosis assay. The inhibition zone was measured in millimeters from the edge of the bacterial colony to the edge of the inhibited fungal mycelia. Mean values followed by different letters show significant difference at $P < 0.05$ using Fischer's protected LSD test.

[b] Mean values for sclerotia germination inhibition. Numbers followed by different letters show significant difference at $P < 0.05$ using Fischer's protected LSD test.

[c] Mean values for disease ratings in detached leaf assay. Numbers followed by different letters show significant difference at $P < 0.05$ using Fischer's protected LSD test.

Table 4. Effect of inoculation technique on disease severity

Technique	Lesion length (%)[a]
Sclerotium	15.46 a
Agar Plug	7.61 b
Mycelial Ball	17.89 a
Control	0 c
LSD 0.05	5.336

[a.] Mean values for total lesion height percentage. Means were compared using Fischer's protected LSD test at P < 0.05. Values followed by different letters are significantly different.

Table 5. Effect of *Paenibacillus* strains on sheath blight disease severity of rice in the greenhouse

Strain	Species ID	Mean lesion length (%)[a]	Mean disease reduction (%)[b]	Disease severity rating[c]
JJ16	*Paenibacillus peoriae*	16.41	13.77	1.42
JJ165	*Paenibacillus peoriae*	17.73	7.33	1.46
JJ195	*Paenibacillus jamilae*	14.54*	23.59	1.31*
JJ228	*Paenibacillus peoriae*	14.22*	25.28	1.50
JJ333	*Paenibacillus jamilae*	14.07**	26.06	1.37
JJ845	*Paenibacillus jamilae*	17.73	6.83	1.50
JJ1580	*Paenibacillus peoriae*	13.99**	26.48	1.27*
JJ1710	*Paenibacillus peoriae*	12.44***	34.63	1.28*
JJ1824	*Paenibacillus jamilae*	11.44***	39.88	1.17**
Control	Inoculated	19.03	-	1.66
Control	Non-inoculated	0	0	0

*** P < 0.0001 ** P < 0.001 * P < 0.01

[a.] Mean values for total lesion height percentages. Values followed by asterisks are significantly different from inoculated control. Means were compared using Dunnett's method with alternative = "less"

[b.] Mean disease reductions were calculated using total lesion height values.

[c.] Mean values for disease severity ratings. Values followed by asterisks are significant. Means were compared using Dunnett's method with alternative = "less"

Figure 1. Antibiosis exhibited by *Paenibacillus* against *Rhizoctonia solani*

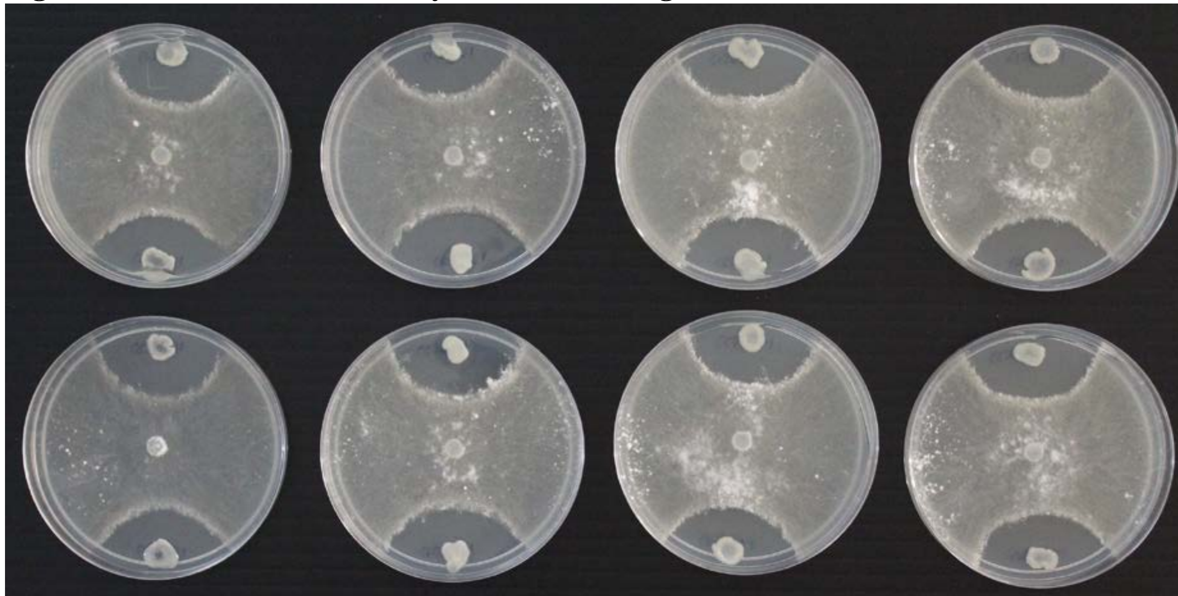
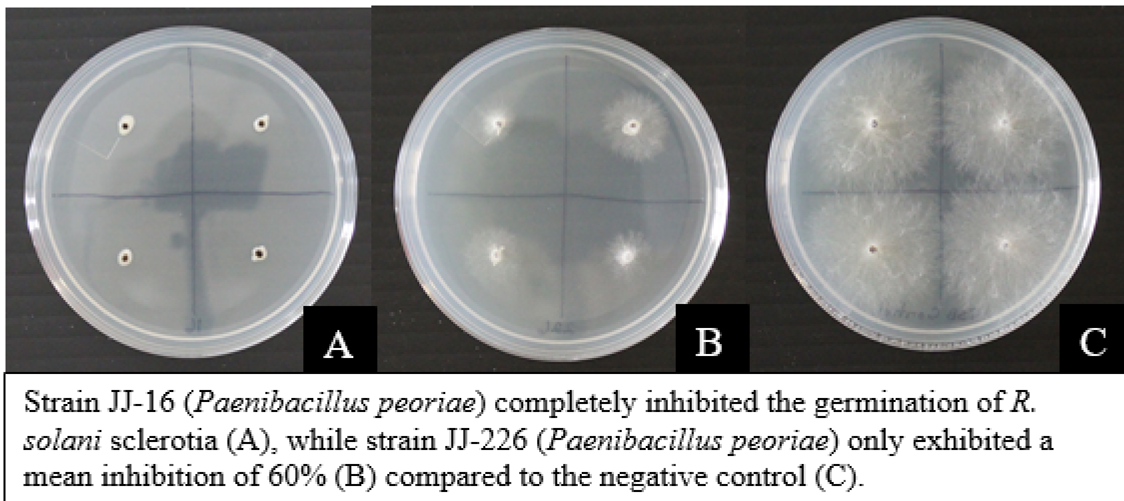
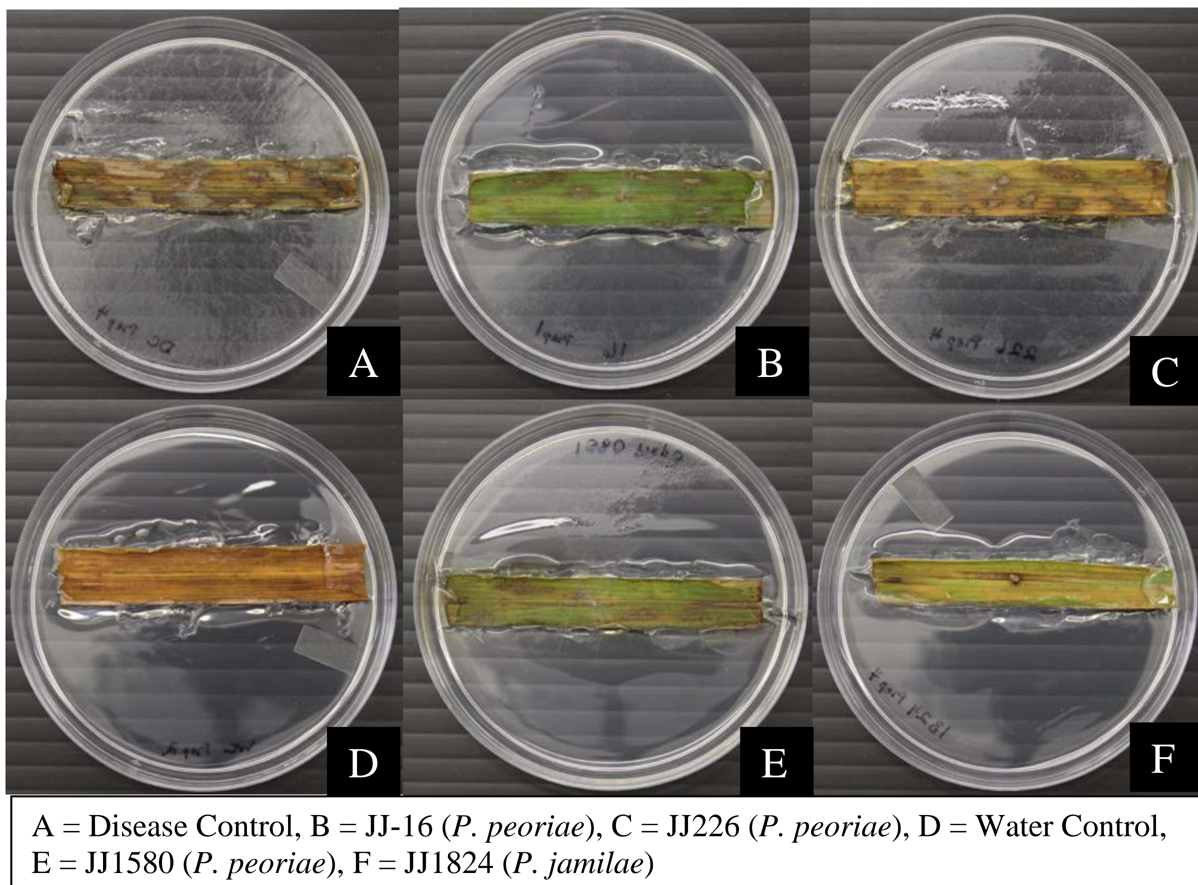


Figure 2. Sclerotia Germination Assay



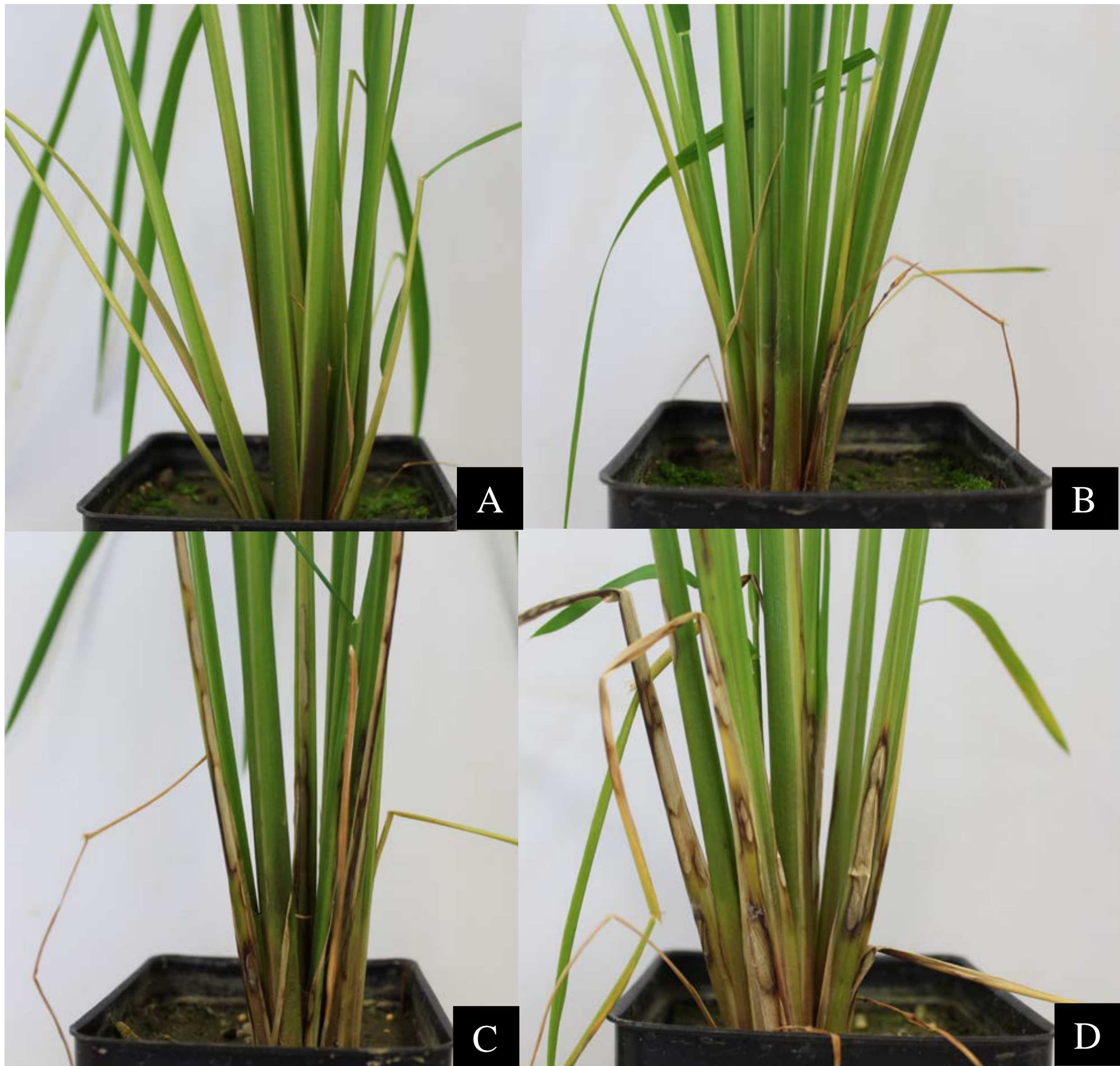
Strain JJ-16 (*Paenibacillus peoriae*) completely inhibited the germination of *R. solani* sclerotia (A), while strain JJ-226 (*Paenibacillus peoriae*) only exhibited a mean inhibition of 60% (B) compared to the negative control (C).

Figure 3. Detached Leaf Assay



A = Disease Control, B = JJ-16 (*P. peoriae*), C = JJ226 (*P. peoriae*), D = Water Control, E = JJ1580 (*P. peoriae*), F = JJ1824 (*P. jamilae*)

Figure 4. Effect of inoculation methods on sheath blight foliar lesion development.



The water control (A) showed no signs of disease. Agar plug inoculation (B) resulted in the lowest mean total lesion height percentage of 7.61, while sclerotium (C) and mycelial ball inoculation (D) had the highest mean total lesion height percentages (15.46 and 17.89 respectively).

Figure 5. Effect of top *Paenibacillus* strains on lesion height percentage in greenhouse

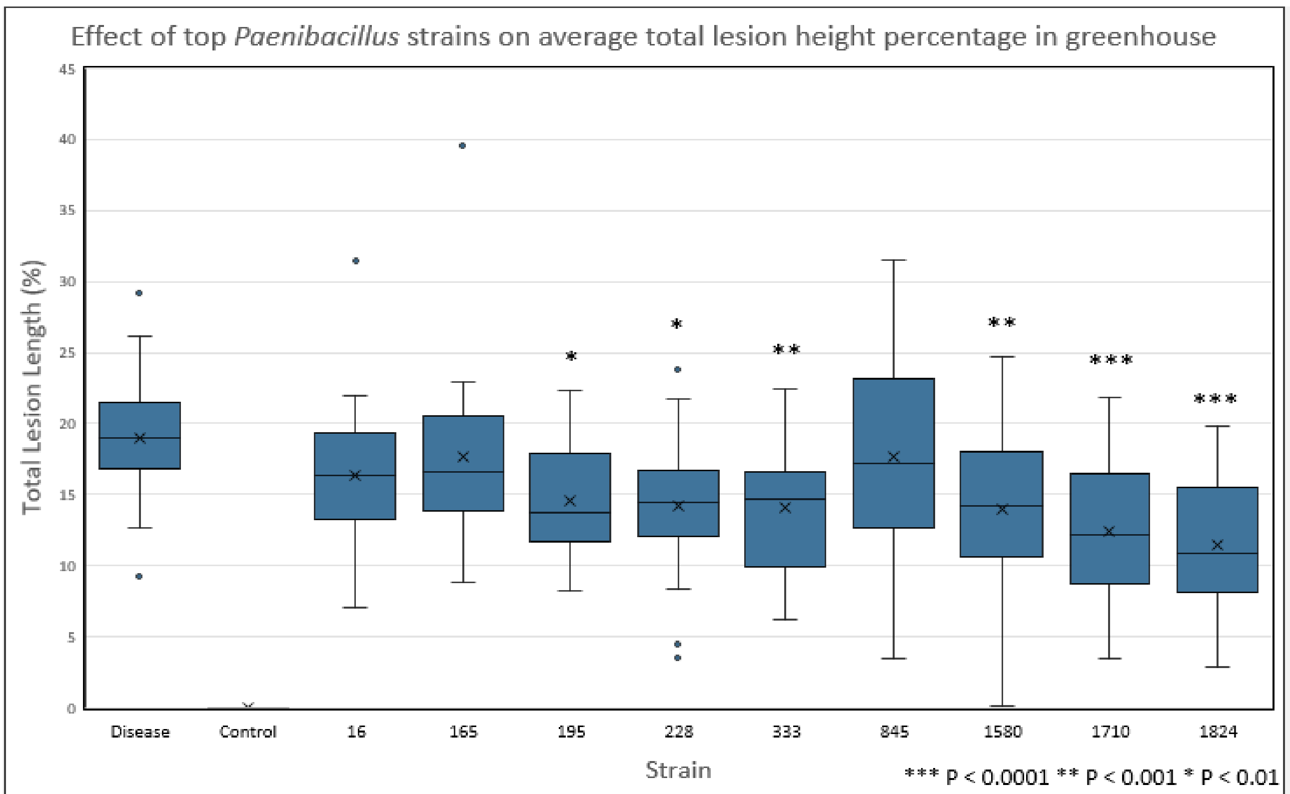


Figure 6. Effect of top *Paenibacillus* strains on average disease rating in greenhouse

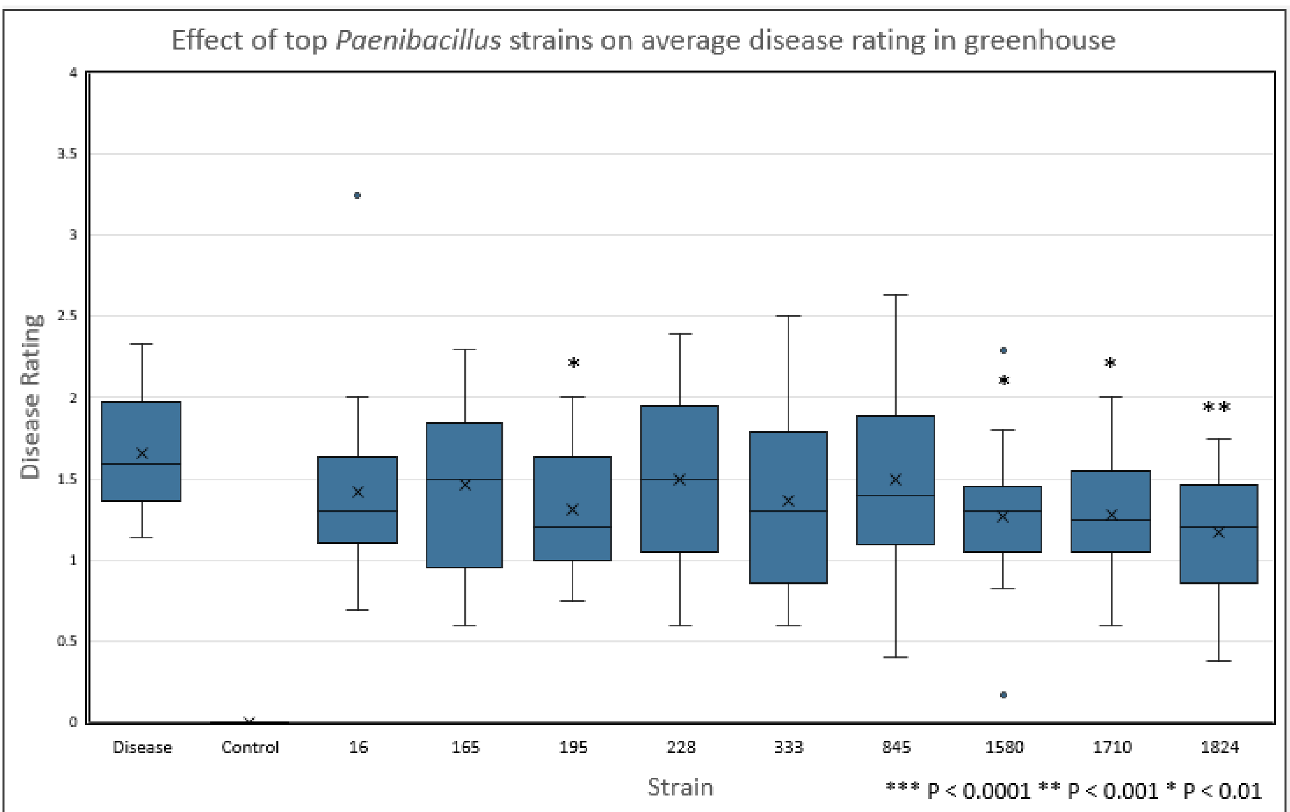
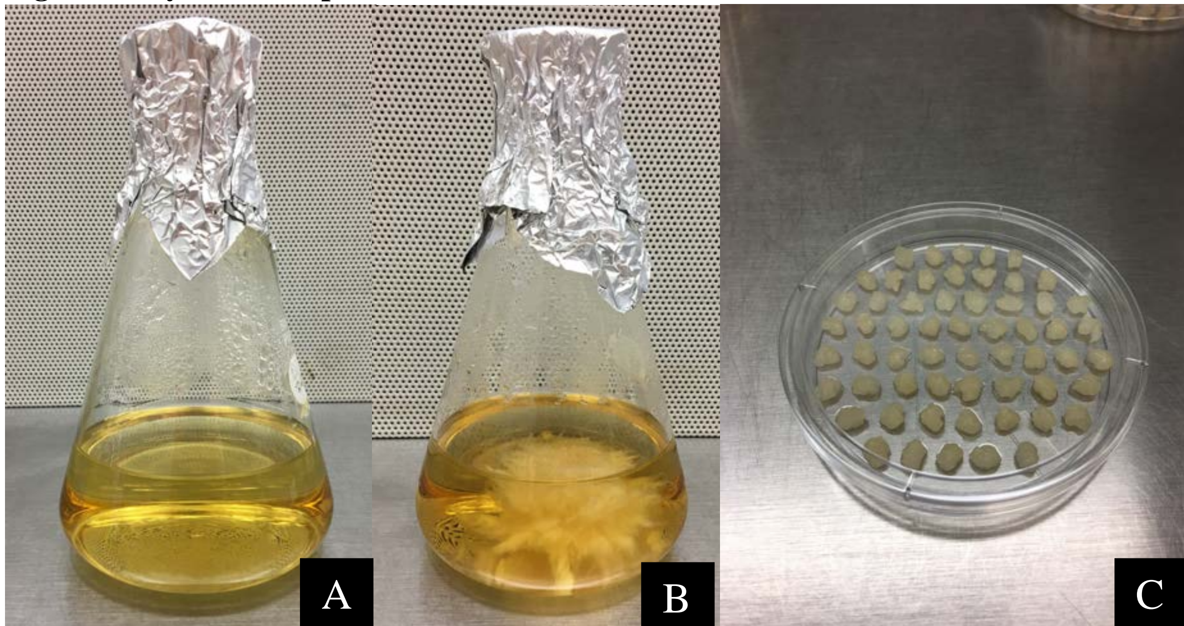


Figure 7. Mycelial ball production



For mycelial ball production, a plug was first inoculated into 150 ml of PDA (A), and was placed on a shaking incubator at 150 rpm at 25℃ for 6-7 days. The resulting ball (B) was then cut into sections, blotted on sterile filter paper, and weighed to 0.1 gram mycelial balls (C).

Chapter 3: Evaluation of *Paenibacillus* strains for their potential to promote early growth of rice in a greenhouse setting

1. Introduction

The use of chemical fertilizers in agriculture is necessary to achieve optimum yields in crops; however most chemical fertilizers applied to plants are not readily utilized and are leached away or fixed to plant-unavailable forms in the soil (Glass, 2003; Gyaneshwar et al. 2002). Additionally the over-application of fertilizers can lead to unfavorable effects on the environment such as eutrophication of water sources (Correll, 1998; Conley et al. 2009) and pollution of the atmosphere through ammonia volatilization and denitrification (Choudhury and Kennedy, 2005).

Inoculation of crop plants with PGPR could offer an alternative to reduce or eliminate the amount of fertilizers applied to crops. PGPR act as biofertilizers through several mechanisms including biological nitrogen fixation, phosphorus solubilization, and the production of siderophores which chelate iron in the soil to a plant usable form (Vessey, 2003). Well-known rhizobia species are symbiotically associated with the roots of legumes and fix atmospheric nitrogen through a nitrogenase enzyme system (Zahran, 2001). Other PGPR such as those from the genera *Azospirillum, Azotobacter, Bacillus* and *Paenibacillus* fix atmospheric nitrogen as well, however they are not symbiotically associated with plant roots (Steenhoudt and Vanderleyden, 2000; Mrkovacki and Milic, 2001; Ding et al. 2005). PGPR help dissolve phosphorus from mineral complexes with iron, aluminum, and calcium by the production of organic acids or through mineralization of organic phosphorus sources, which in turn makes phosphorus available to the plant (Trolove et al. 2003; Sharma et al. 2013).

The objective of this study was to evaluate *Paenibacillus* strains previously demonstrating antagonism to *R. solani* for their potential to promote early growth of rice. The rationale was to try to identify strains exhibiting multiple modes of plant growth promotion.

2. Materials and Methods

2.1 PGPR Culture

In this experiment *Paenibacillus* strains previously exhibiting antagonism *in vitro* to *Rhizoctonia solani* were assessed for their potential to promote early growth of rice. Strains were previously identified using 16S rDNA sequencing. Prior to use, all strains were stored in tryptic

soy broth amended with 30% glycerol at -80ºC. Each strain was then taken from cold storage, streaked onto TSA plates, and incubated at 28ºC for 48 hours and then checked for purity. Bacterial suspensions to be used for *in planta* assays were quantified using the McFarland standards (McFarland, 1907) and checked using serial dilutions.

2.2 Greenhouse Colonization Experiment

An early growth promotion assay was performed in conetainers filled with field soil. Fine sandy loam field soil (80% sand, 10% silt and 10% clay) supplied from E.V. Smith Research Station (located in Shorter, Alabama) was first pasteurized before using for growth promotion assays. Soil was pre-moistened before loading into the pasteurizer. Once loaded, the soil was pasteurized at 88°C for 9 hours and then allowed to sit overnight to allow for the germination of any dormant fungal propagules. Soil was then pasteurized again for an additional 9 hours and then removed and stored in plastic bins prior to use. Conetainers with a diameter of 7.5 cm and height of 24.5 cm were plugged with a cotton ball before filling with 750 grams of pasteurized field soil. Rice cv. CL111 seeds were first pre-germinated by incubating in moistened paper towel for two days before using for growth promotion assays. After two days, only rice seeds showing signs of germination were used in experiments. One pre-germinated rice seed was then planted 2.5 cm deep in a single conetainer. One milliliter of respective bacterial treatments at an approximate concentration of 10^6 cfu/ml were applied to the top of the seed before covering. Bacterial suspensions were made by scraping plates of pure culture bacteria and mixing with sterile water. Conetainers were then watered, and plants were allowed to grow for 4 weeks before destructively sampling. Plant parameters evaluated included shoot height in millimeters, tiller number, and dry shoot and root weight measured in grams. Roots were carefully removed from soil and washed thoroughly before drying. Plant tissue samples were dried for 48 hours at 70°C before weighing.

2.3 Statistical Analysis and Experimental Design

Experiments were setup as a randomized complete block design (RCBD) with 10 replications per treatment, and greenhouse trials were repeated twice to validate results. Since twenty-one strains were screened per trial, trials were broken into three test – each consisting of seven bacterial treatments and one water control. Results from the three trials were pooled for statistical analysis. No statistical significance was observed when trials were analyzed separately. All data were analyzed by analysis of variance (ANOVA) to test for significance at the ($p \leq 0.05$)

using R statistical analysis software. Treatment means were then separated using Dunnett's method at $p \leq 0.05$ with treatment groups being compared to water control using the package "multcomp" in R statistical analysis software.

3. Results

3.1 Greenhouse Colonization Experiment

Overall, twenty of the *Paenibacillus* strains did not significantly promote any of the selected plant growth parameters: shoot height, tiller number, and dry shoot and root weight (Table 5). One *Paenibacillus polymyxa* strain (JJ1823) significantly increased the amount of tillers on the rice plant; however, it did not significantly increase plant height or dry root and shoot weight.

4. Discussion

The inability of *Paenibacillus* strains to promote the growth of rice plants could be a reflection of the fact that they were chosen based on their biocontrol phenotype, rather than their phenotypes relating to plant growth promotion (ie. nitrogen fixation, phosphorus solubilization, etc.). The rationale of using strains previously demonstrating disease biocontrol was to try and identify strains exhibiting multiple mechanisms of plant growth promotion. *P. polymyxa* strains are known to exhibit a wide range of phenotypes relating to growth promotion of different crops including IAA (Erturk et al. 2010) and siderophore production (Padda et al. 2017), nitrogen fixation (Puri et al. 2016), and phosphotase production (Xu and Kim, 2014); however, the strains of *P. polymyxa* selected for this study did not promote the early growth of rice plants. One exception was *P. polymyxa* strain JJ-1823, which significantly increased the average number of rice tillers. Reports on the potential of *P. peoriae* strains to promote the growth of plants are sparse; however, some strains have been shown to produce exogenous IAA and phosphatase (Xu and Kim, 2014). Reports of plant growth promotion by *P. jamilae* seem to be missing altogether.

Table 6. Early growth promotion of *Paenibacillus* strains on rice shoot height, tillers, and dry shoot and root weights.

Strain	Species ID	Shoot height (mm)[a]	Tiller number[b]	Dry shoot weight (g)[c]	Dry root weight (g)[d]
JJ16	*P. peoriae*	504.00	1.23	0.304	0.187
JJ165	*P. jamilae*	486.70	1.27	0.292	0.171
JJ195	*P. peoriae*	484.27	1.03	0.285	0.176
JJ226	*P. peoriae*	473.40	1.27	0.310	0.184
JJ227	*P. peoriae*	482.27	1.03	0.272	0.163
JJ228	*P. peoriae*	472.63	1.37	0.279	0.153
JJ333	*P. jamilae*	496.77	1.30	0.321	0.206
JJ845	*P. jamilae*	475.83	1.17	0.251	0.140
JJ1580	*P. peoriae*	496.07	1.62	0.293	0.168
JJ1614	*P. peoriae*	475.21	1.18	0.261	0.163
JJ1640	*P. peoriae*	493.33	1.20	0.284	0.151
JJ1642	*P. peoriae*	479.23	1.27	0.259	0.159
JJ1706	*P. peoriae*	481.77	1.23	0.289	0.178
JJ1710	*P. peoriae*	489.47	1.40	0.297	0.164
JJ1720	*P. peoriae*	472.40	1.50	0.305	0.144
JJ1724	*P. peoriae*	455.80	1.27	0.253	0.138
JJ1743	*P. polymyxa*	458.47	1.50	0.296	0.151
JJ1747	*P. polymyxa*	436.50	1.30	0.261	0.157
JJ1749	*P. peoriae*	471.57	1.60	0.328	0.185
JJ1823	*P. polymyxa*	481.87	1.77*	0.324	0.204
JJ1824	*P. jamilae*	456.43	1.50	0.295	0.176
Control		477.47	1.17	0.271	0.175

*** P < 0.0001 ** P < 0.001 * P < 0.01

[a.] Mean values for shoot height. Values followed by asterisks are significant. Means were compared using Dunnett's method with alternative = "greater"

[b.] Mean values for tiller number. Values followed by asterisks are significant. Means were compared using Dunnett's method with alternative = "greater"

[c.] Mean values for dry shoot weight. Values followed by asterisks are significant. Means were compared using Dunnett's method with alternative = "greater"

[d.] Mean values for dry root weight. Values followed by asterisks are significant. Means were compared using Dunnett's method with alternative = "greater"

Chapter 4: Colonization dynamics of three selected *Paenibacillus* strains in the rice phyllosphere

1. Introduction

Paenibacillus species are frequently found in association with plants, and have been isolated from diverse plant tissues including coffee cherries (Sakiyama et al. 2001), turmeric rhizomes (Aswathy et al. 2012), and potato leaves and roots (Krechel et al. 2002). Strains of *P. polymyxa* specifically have been shown to endophytically colonize root, stem, and leaf tissue of lodgepole pine (Anand et al. 2013), and have been found in association with roots of cucumber (Park et al. 2004), barely (Timmusk et al. 2011), peanut (Haggag, 2007), sesame (Ryu et al. 2006), corn (Von der Weid et al. 2000), and watermelon (Yaoyao et al. 2017). While the production of antimicrobial compounds is important in disease suppression by PGPR, the ability of a PGPR strain to effectively colonize plant tissues is often related to its ability to be an efficient biocontrol agent (Haggag and Timmusk, 2008; Timmusk et al. 2005).

Since several *Paenibacillus* strains were shown to significantly reduce the amount of disease caused by *R. solani in planta* it was of interest to us to evaluate the population dynamics of some strains in the rice phyllosphere after foliar inoculation. Three strains (JJ-165, JJ-1710, JJ-1823) were chosen as representative members of each species for rifampicin marking with the rationale of using a strain that performed very well (JJ-1710), a strain that performed moderately well (JJ-165), and a strain that did not perform well (JJ-1823) to evaluate if their population dynamics differed in the rice phyllosphere and if this difference could have played a role in their performance in suppressing the amount of disease caused by *R. solani*.

2. Materials and Methods

2.1 Rifampicin marking of bacteria

A stock solution of rifampicin was first made by dissolving 1.0 gram of rifampicin in 100 ml of dimethyl sulfoxide (DMSO). The solution was then filter sterilized and stored in a dark-brown, sterile glass container, since rifampicin is photo-sensitive, and stored in the refrigerator. Since DMSO freezes in the refrigerator the solution was set out several hours before use to thaw. Rifampicin-resistant mutant bacteria were made by first inoculating 250 ml Erlenmeyer flasks filled with 100 ml of TSB with respective strains. The strains were then grown for 24 hours on a shaking incubator at 150 rpm maintained at 25ºC. After 24 hours each flask was injected with 1.0

ml of rifampicin stock solution (100 ppm), covered with aluminum foil, and incubated for an additional 6 days. Broth cultures were sampled from at 2, 3, 4 and 6 day time points and 100 µl were plated on TSA plates amended with rifampicin (RTSA) at a rate of 100 ppm. RTSA plates were made by amending TSA with 1.0 ml of rifampicin stock solution per 100 ml of TSA.

Colonies that grew on RTSA were considered resistant to rifampicin at a rate of 100 ppm. Several rif-mutants were isolated for each strain and their colony morphology and growth rate were visually compared to the type strain. A single mutant was chosen per strain and only mutants exhibiting similar growth rate and colony morphology were used as representatives for type strains.

2.2 Greenhouse Colonization Experiment

Square, black pots measuring 10.5 cm across and 12.5 cm deep were used in this study. The bottom of the pots were wrapped with black landscape fabric and secured using clear packing tape to prevent soil from leaking out during flooded conditions. Pots were then filled with 1300 grams of mineral field soil obtained from E.V. Smith Research Station located in Shorter, Alabama. Phosphorus and potassium fertilizers were amended into the soil at this stage following the recommendation of a soil test. Three seeds of the rice cultivar CL111 were sowed per pot to a depth 2.5 cm and hand-watered to field capacity until the seedlings had reached fourth leaf stage. At the fourth leaf stage, the strongest plant per pot was kept and the rest were thinned so that only one plant remained. Each pot was then placed into its own individual clear, round plastic container measuring 16.8 cm across and 11 cm deep. Nitrogen fertilizer was applied at this stage in the form of urea (44-0-0) following the recommendation of a soil test.

Respective bacterial suspensions were then applied as foliar spray using a hand sprayer to rice plants until just before runoff, making sure to thoroughly cover leaves, leaf undersides, and sheaths. Rif mutant bacterial suspensions were made by scraping bacteria grown on RTSA and suspending in water to a final approximate concentration of 10^8 cfu/ml. Water replacing bacterial suspensions served as a control. Rice plants were sampled at three time points after application: immediately after, 1 week, and 2 weeks after. Each test contained four treatments (1 water control and 3 bacteria), and each treatment had seven replications. Plants were sampled from at respective time points by removing one rice tiller per plant and placing into individual bags for transportation to the lab. Equipment was sterilized between treatments. In the lab, tillers were removed from their bags and were cut and weighed into 1.0 gram samples (0.5 grams leaf tissue

and 0.5 grams stem tissue) and were considered representative for plant replications. Samples were then grinded separately using a Kleco for 90 seconds by placing into metal canisters containing a metal ball. Once grinded, 10 ml of sterile water were added to each canister and were homogenized by shaking for 30 seconds. Homogenized samples were then transferred to 15 ml tubes and were serially diluted. Dilutions 10^{0}, 10^{-2}, and 10^{-4} were plated for each replication onto I-plates measuring 15 mm in diameter and containing half TSA and half RTSA. Dilutions were plated in duplicate for each dilution and 25µl were plated per each side of I plate and spread using sterile loops. Plates were then incubated for 72 hours at 28ºC and colonies were counted. This process was repeated for each time point. The test was repeated once to validate results and tests were setup as a completely randomized design (CRD).

2.3 Statistical Analysis and Experimental Design

A greenhouse experiment consisting of four treatments (three rifampicin-resistant mutant strains and a water control) was arranged in a completely randomized design (CRD). Treatments contained six replications and the greenhouse experiment was repeated once to validate results. Results from the two experiments were pooled for statistical analysis. All data were analyzed by analysis of variance (ANOVA) using R statistical software to test for significance at the critical value ($P \leq 0.05$). Treatment means were then compared using Fischer's protected LSD test at $p \leq 0.05$ using the "agricolae" package and letters of significance were assigned using the least significant difference.

3. Results

3.1 Rifampicin-resistant Bacteria

Several rif mutants were generated per strain through co-culturing with rifampicin in broth culture and then plating onto RTSA. Overall, 5 mutant strains of *P. jamilae* JJ-165 were generated (165rif1, 165rif2, 165rif3, 165rif4, 165rif5), 5 strains of *P. peoriae* JJ-1710 (1710rif1, 1710rif2, 1710rif3, 1710rif4, 1710rif5), and 6 strains of *P. polymyxa* JJ-1823 (1823rif1, 1823rif2, 1823rif3, 1823rif4, 1823rif5, 1823rif6). Strains 1823rif2 and 1823rif3 were later discarded since their colony morphologies were not similar to that of the type strain. Mutant strains 165rif1, 1710rif2, and 1823rif4 were chosen for colonization studies in greenhouse experiments based on their similar growth rate and colony morphology to the type strains.

3.2 Greenhouse Experiment

After initial application of bacterial suspensions (approximately 1 x 10^8 cfu/ml), samples for the immediate time point were taken. Overall, all mutant strain populations drastically decreased upon introduction and re-isolation from the plant with mutant strains 165rif1, 1710rif2, and 1823rif4 decreasing from the initial population size by 4-fold, 5-fold, and 4-fold respectively. Strain 165rif1 (*P. jamilae*) had higher populations across time points 0 and 1, however the population size in general was very low. Strain 1710rif2 (*P. peoriae*) had the lowest population size at all time points, which is surprising since it was among the top two performing strains in the rice disease greenhouse assay. 1823rif4 (*P. polymyxa*) maintained the second largest population size through all time points; however, no strains were statistically significant from each other at the 2 week time point.

After spreading and incubating homogenized plant tissue samples on TSA and RTSA, far fewer colonies of the mutant strains were recoverable on the RTSA side than the TSA side. For this reason, only bacterial colonies on the TSA side were counted to give the most accurate amount of bacteria present in plant tissue samples. This phenotype was termed by McInroy et al. as "antibiotic masking" (McInroy et al. 1996). The authors suggest that expression of antibiotic-resistance is altered *in planta* and that this masking accounts for the inability of rifampicin-resistant strains to grow on RTSA upon isolation from plant tissues. Mutant strains growing on the TSA side still retain their antibiotic resistance and grow when transferred to RTSA. For confirmation that the strains we were counting were indeed our mutant strains, they were transferred from the TSA side of the I-plate to RTSA to confirm antibiotic-resistance. Other than the mutant strains, very few other bacteria were isolated from rice tissue samples.

4. Discussion

The use of rifampicin-resistant bacteria is a common approach to evaluating the colonization dynamics of a selected PGPR (Sheng and Xia, 2006; Ryu et al. 2007; Maldonado et al. 2011), although this technique is more commonly applied to soil systems. Overall, all bacterial populations reduced steadily over a two week period after initial application which would suggest that strains did not establish and proliferate in the phyllosphere. This is not surprising since the phyllosphere is a much harsher environment than the rhizosphere. Bacteria in the phyllosphere are subject to fluctuations in water and nutrient availability, are exposed to ultraviolet radiation, and may be suppressed by plant defense compounds (Vorholt, 2012). For these reasons bacterial abundance in the phyllosphere if often lower in comparison to that of the

rhizosphere (Delmotte et al. 2009). While strains of *P. peoriae* and *P. jamilae* have not been isolated from the phyllosphere, other strains of *Paenibacillus* have been found in association with plant leaves (Krechel et al. 2002) and stems (Anand et al. 2013); however, differences in the ability of strains of the same species to colonize plant tissues have been observed in soil systems (Shishido et al. 1996). The inability of strains to proliferate in the phyllosphere could also be a consequence of their original isolation from the rhizosphere, for which they may be more adapted.

Table 7. Colonization dynamics in the rice phyllosphere

Mutant Strain	Species ID	Inoculant Population (LOG)[a]	Week 0 Population[b]	Week 1 Population[c]	Week 2 Population[d]
165rif1	*P. jamilae*	8.0	3.94 a	2.47 a	0.63 a
1710rif2	*P. peoriae*	8.0	2.88 b	1.08 b	0.21 a
1823rif4	*P. polymyxa*	8.0	3.11 b	1.90 ab	0.28 a
Control	-	0.0	0.00 c	0.00 c	0.00 a
LSD$_{0.05}$	-	-	0.36	1.01	0.69

a. Values represent initial bacterial population during time of application. Values were LOG transformed.

b. Values represent mean bacterial population size in cfu/gram of fresh tissue at the immediate sampling time point after inoculation. Values were log transformed and numbers followed by different letters show significant difference at $P < 0.05$ using Fischer's protected LSD test.

c. Values represent mean bacterial population size in cfu/gram of fresh tissue at the one week sampling time point. Values were log transformed and numbers followed by different letters show significant difference at $p < 0.05$ using Fischer's protected LSD test.

d. Values represent mean bacterial population size in cfu/gram of fresh tissue at the two week sampling time point. Values were log transformed and numbers followed by different letters show significant difference at $p < 0.05$ using Fischer's protected LSD test.

Figure 8. Comparison of mutant Rif strains to type strains

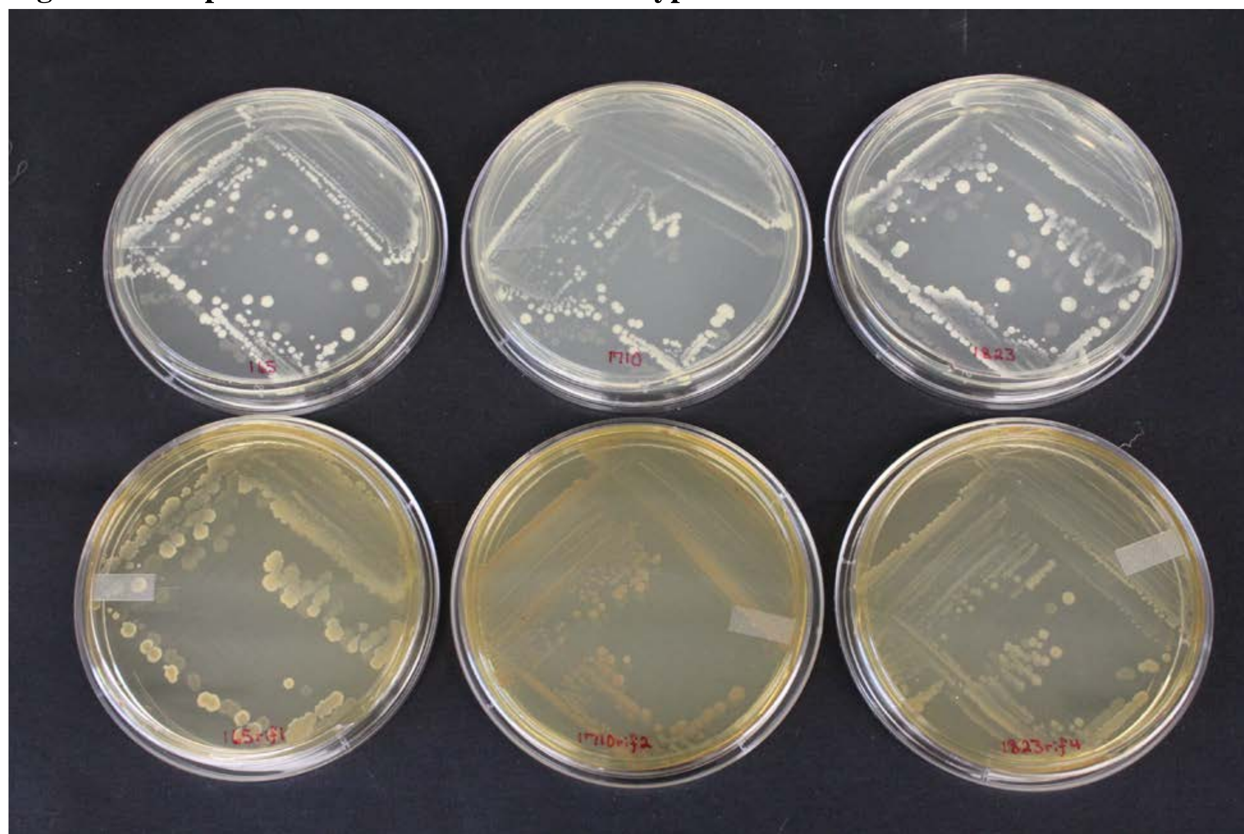
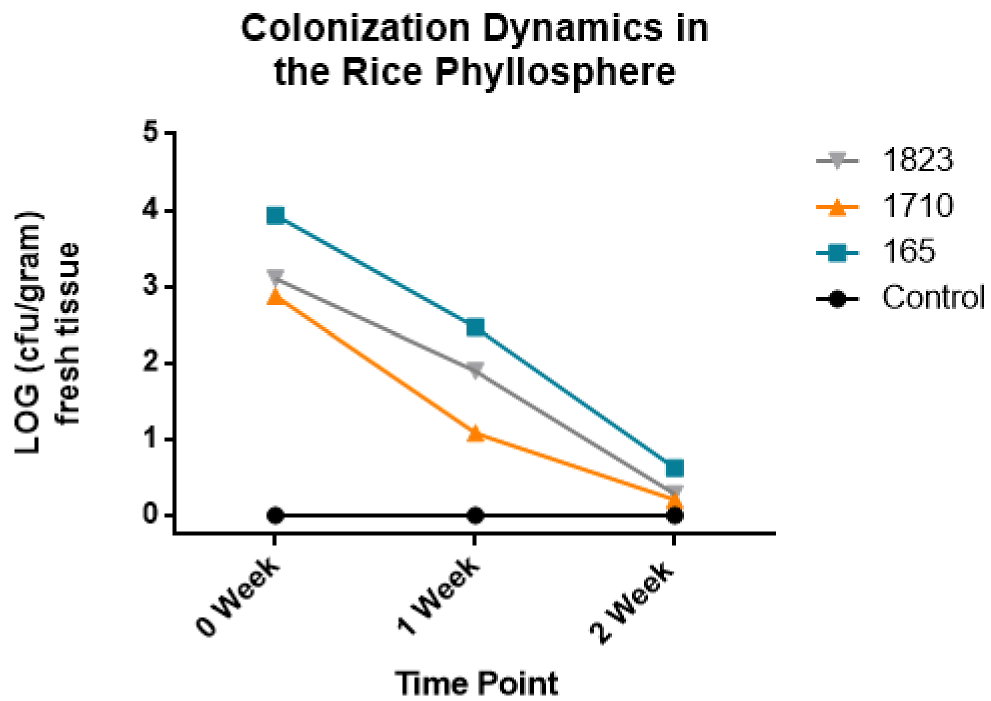


Figure 9. Gene-masking in rifampicin-resistant mutants

Figure 10. Bacterial colonization of the rice phyllosphere



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