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DEVELOPMENT OF BIOLOGICAL TOOLS TO PROMOTE RICE HEALTH AND GROWTH

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science

in

The Department of Plant Pathology and Crop Physiology

by
Ateet Maharjan
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ABSTRACT

Sheath blight (ShB - caused by *Rhizoctonia solani*) and bacterial panicle blight (BPB - caused by *Burkholderia glumae*) are economically important rice diseases in Louisiana and other rice-growing regions. Fungicides and oxolinic acid are used to manage ShB and BPB, respectively, but these chemical methods are not sustainable economically and ecologically. Besides diseases, plant utilizable nitrogen (N) in the soil is inadequate for optimum crop yield, leading to the use of inorganic fertilizers and making agriculture less sustainable. To develop alternative biological control methods for protecting rice plants from diseases, rice-associated bacteria were screened based on their antagonistic activities against the pathogens by growth-inhibition plate assays. Three strains each of *Bacillus* spp. and *Pseudomonas* spp. were selected for evaluation of their disease suppression activities against ShB and BPB, respectively. Rice plants were artificially inoculated with *R. solani* at the tillering stage, while *B. glumae* was inoculated at 30% heading separately. Selected antagonistic bacteria were applied via spraying on the sheath (for ShB) or panicles (for BPB) of plants 24 h after pathogen inoculation. Efficacy of the *Pseudomonas* strains for suppression of BPB could not be determined in the field trials because of low disease pressure. Whereas, field trials in 2017 and 2018 revealed that foliar spray of *Bacillus* strain REB711 significantly reduced ShB development compared to the non-treated control, although it was less effective than the fungicide azoxystrobin. Seed treatment with *Bacillus* strain REB711 significantly reduced ShB development in the greenhouse environment, which could result from competition, antibiosis, and/or induction of plant defense system. To identify bacterial agents for rice growth promotion, bacteria isolated from the rice rhizosphere were screened based on their nitrogen-fixing activity, and five isolates were selected to test their ability to promote rice growth at an early seedling stage in the laboratory and greenhouse
conditions. Of five selected isolates, seed treatment with the *Pseudomonas* strains RRB I-6 seemed to be potential in promoting the growth of rice seedlings. Increases in plant height and soil-plant analysis development (SPAD) scores could have resulted from increased uptake of N or other nutrients or production of phytohormones. These results indicate that *Bacillus* sp. REB711 and *Pseudomonas* sp. RRB I-6 could be potential biological agents for managing ShB and promoting rice growth, respectively.
CHAPTER I. INTRODUCTION AND LITERATURE REVIEW

1.1. Bacterial panicle blight (BPB) disease of rice

Bacterial panicle blight (BPB) is one of the potential threats to rice production worldwide and in the southern United States, including Louisiana. It was first described as grain rot and seedling blight of rice in Japan in 1956 (Goto and Ohata, 1956; Kurita and Tabei, 1967). Since then, it has been reported in other rice-growing countries of Asia, Africa, South and Central America (Chien and Chang, 1987; Cottyn et al., 2001; Zeigler and Alvarez, 1989). In the United States, panicle blight was established as one of the significant issues of rice in the mid-1990s with the introduction of susceptible varieties, such as Bengal, Cypress, and Cocodrie (Wamishe et al., 2014). The cause of panicle blight had been initially attributed to unknown physiological factors until the scientists of the Louisiana State University Agricultural Center identified Burkholderia glumae and B. gladioli as the causal agents in 1996/97 (Groth et al., 1991; Nandakumar et al., 2009; Shahjahan et al., 2000). Severe BPB can result in sterile panicles, grain abortion and grain rot leading to yield reduction by 75% (Trung et al., 1993). The disease can be problematic with the change in climate favoring disease development (Schaad, 2008) and has increased recently due to the genetic diversity of the pathogen (Karki et al., 2012; Seo et al., 2015) and lack of effective management practices.

1.1.1. Pathogen

BPB is mainly caused by B. glumae; however, B. gladioli has also been reported to cause similar symptoms of panicle blighting (Nandakumar et al., 2009). B. glumae is more virulent, damaging, and frequently isolated from BPB affected fields compared to B. gladioli (Mulaw et al., 2018; Nandakumar et al., 2009). B. glumae is a gram-negative, rod-shaped and non-fluorescent bacteria with lophotrichous flagella (Ham et al., 2011). B. glumae is a seed-borne pathogen; however, its presence in bulk soils of crop zone was also reported (Uematsu et al.,
B. glumae produces toxoflavin, a phytotoxin responsible for its virulence activity (Kim et al., 2004).

1.1.2. Symptoms

![Figure 1.1](image_url)

**Figure 1.1.** Symptoms of BPB on rice panicle in the field. Two-tone discoloration on panicles (A). Upright panicle infected with B. glumae (B).

Typical symptoms of BPB can be observed as seedling blight, sheath rot, and panicle blighting, which occurs during grain filling reproductive phase and is the principal cause of yield reduction (Nandakumar et al., 2009). Symptoms on panicles include blighted florets having two tones of white/gray discoloration on the base of glumes with brown margin (Figure 1.1A), which later turns grayish black due to bacterial/fungal growth on the surface (Zhou, 2019). Heavily infected panicles are found upright (Figure 1.1B) in the field as BPB hinders the process of grain formation making it light-weighted. The panicles may also develop a dark brown lesion extending from flag leaf sheath (Ham et al., 2011). BPB can cause seedling rot or seedling blight
and also the death of seedlings (Goto and Ohata, 1956), but this phase of the disease has rarely been reported in the field in the southern United States. The symptoms of BPB might be confused with insect damage, water stress, or other environmental factors (Wamishe et al., 2014).

1.1.3. Disease cycle and epidemiology

The disease cycle and epidemiology of BPB has yet to be fully understood. *B. glumae* is a seed-borne pathogen but it can survive in the soil and on the surface of the host plant (Compant et al., 2008). The pathogen seems to invade germinated seeds, inhabit plant surfaces and extend upward along with plant growth infecting the panicles at the flowering stage under favorable conditions (Hikichi, 1993b; Tsushima, 1996). Spreading of bacteria can be primarily made by direct contact with severely diseased panicles and is assisted by splashing rainwater (Tsushima and Naoto, 1991). Favorable environmental factors for BPB include high night temperatures and high humidity accompanied by frequent rainfall. Occurrence of these conditions during flowering and heading stages tend to especially favor the development of BPB in the field (Cha et al., 2001). Severe outbreaks of the disease occurred during 1995, 1996 and 1998 causing yield losses up to 40%, and significant yield losses have been reported during 2000, 2010 and 2011 in Louisiana and other areas in the southern United States (Shahjahan et al., 2000; Wamishe et al., 2014). The outbreaks of BPB in the southern United states causing massive damage were associated with prolonged hot summer nights (Nandakumar et al., 2009).

1.1.4. Disease management

The genetic diversity within the pathogen species and the lack of effective control methods increase the potential of BPB to be an epidemic disease under favorable weather conditions (Karki et al., 2012; Nandakumar et al., 2009). Various cultural, chemical, biological and host resistance strategies have been employed for managing BPB but have not been
satisfactory. Since infected seeds serve as the primary source of inoculum, farmers are recommended to use pathogen-free certified seeds in the United States; however, a system for detection of the pathogen in those seeds has not been employed (Zhou, 2019). Cultural practices, such as proper water management, avoidance of excessive rates of nitrogen fertilizer, optimal seeding rates and early planting, have been studied for years to reduce the damage from BPB but still have not been successful (Wamishe et al., 2014). Likewise, hot water seed sterilization, which was established against rice blast disease, is not successful against *B. glumae* (Hayasaka et al., 2001). On the other hand, resistant varieties would have been a valid option, but only partially resistant varieties like Jupiter and LM-1 are currently available, and they do not have enough desirable agronomic features (Groth et al., 2007; Ham and Groth, 2011). Oxolinic acid, a quinoline derivative, has been used to manage BPB via seed treatment or foliar spray in Japan (Hikichi, 1993a), yet it is not registered in the United States. Moreover, some strains of *B. glumae* have developed resistance against oxolinic acid (Maeda et al., 2004). Copper compounds possessing the antibacterial property have been tested against *B. glumae* (Cui et al., 2014); however, their phytotoxic effects on the rice plants restrict the application of copper products. Lack of effective BPB management options along with adverse impacts of chemical options to human health and environment and the chance of development of resistant strains require development of effective and sustainable options like biocontrol agents to manage BPB.

1.2. **Sheath blight (ShB) disease of rice**

Sheath blight is one of the most destructive diseases worldwide in rice, causing significant grain yield and quality losses. The disease is most prevalent in temperate and tropical rice growing areas adopting intensive rice cultivation. Its occurrence was first described by Miyake from Japan in 1910 and was later reported as ‘oriental leaf and sheath blight’ due to its establishment in Asian rice-growing regions (Kozaka, 1975; Ou, 1985). With the introduction of
semi-dwarf high yielding varieties in the 1970s, and increased application of nitrogen fertilizer accompanied by reduced tillage and short crop rotation, the disease was rapidly established as one of the economically significant rice diseases in Louisiana and other rice-growing regions in the southern United States (Groth and Lee, 2003; Lee and Rush, 1983). In the absence of protection strategies, ShB has resulted in grain yield losses ranging from 4 – 50 % under favorable conditions (Groth et al., 1991; Marchetti and Bollich, 1991). In the rice fields planted with susceptible varieties, severe infection can occur in the entire leaf sheath and leaf blades resulting in a reduction of yield by 50% (Lee and Rush, 1983). Usually, economic damage is from the reduction in quality and quantity of grain yield, milling, harvestability, reduced ratoon production and the cost of fungicide application (Saichuk et al., 2014).

1.2.1. **Pathogen**

*Rhizoctonia solani* Kühn of the anastomosis group AG 1-IA, a filamentous basidiomycete anamorph fungus, causes ShB and is also known by its teleomorph stage *Thanatephorus cucumeris* (A.B. Frank) Donk (Ou, 1985). It is a soil-borne necrotrophic fungus with the ability to attack a wide range of hosts, including soybean, potato, sorghum and grassy weeds (Lehtonen et al., 2007). *R. solani* can survive as a dormant mycelium and as overwintering structures called sclerotia in the soil or host debris for many years. The colorless vegetative mycelia turn brown with maturity and hyphae branched at 90-degree angle can be observed under a microscope.

1.2.2. **Symptoms**

*R. solani* can infect host plants throughout the entire growth stages beginning with seedlings, yet, in the United States, development of ShB infection is observed only in the late tillering to panicle differentiation stages (Rush and Lee, 1992). The characteristic early symptoms include 1 – 3 cm long oval or ellipsoidal water-soaked lesions on the leaf sheath near the soil/water level (Figure 1.2A). Under conducive environments, lesions multiply, join, extend
upward from the sheaths to leaf blades, and spread laterally to the nearby plants in contact through runner hyphae in a dense planting condition (Lee and Rush, 1983). The fungus may spread to the flag leaf sheath and panicles (Figure 1.2C) and causes lodging of the plants by loosening the sheath from culm under severe conditions (Ou, 1985). Irregular lesions on the leaf, later on, turn greenish gray and grayish white spots with brown margin resembling necrotic regions. Coalescence of multiple dry lesions on the leaf gives the appearance of snakeskin (Figure 1.2B). The infected tissue produces white sclerotia, which on maturing turn hard and brown and are easily detached from the infected surface (Lee and Rush, 1983).

**Figure 1.2.** Symptoms of sheath blight on rice plants. Symptoms on rice sheath above the water line (A), on the leaf (B) and the flag leaf and panicles (C).
1.2.3. **Disease cycle and epidemiology**

Dormant sclerotia in the soil and mycelia on crop residues are the primary inoculum source for initiation of infection cycle each cropping season (Hori and Anraku, 1971). Sclerotia or plant debris from the previous growing season comes into physical contact with rice culms floating on the water surface of flooded rice fields. The fungus penetrates and colonizes tissues on the sheath developing a lesion which, later on, extends vertically to the upper leaf sheath, leaf blades and flag leaf (Rush and Lee, 1992). The fungus also spreads horizontally to the nearby tillers and leaves in contact through runner hyphae. Susceptible semi-dwarf long-grain varieties grown in closer plant spacing under low light, warm temperature (28 – 32 °C) and high relative humidity of crop canopy (85 – 100%) accelerate development and spreading of the fungus (Castilla *et al.*, 1996; Lee and Rush, 1983). ShB infection usually occurs in a circular pattern in the field resembling a bird’s nest structure, and its timing contributes to high yield loss (Saichuk *et al.*, 2014). The initial amount of inoculum, plant growth stage, environmental factors, host resistance, and cultural management have significant effects on the development of rice sheath blight and grain yield (Boyette and Lee, 1979; Groth and Bond, 2007; Ou, 1985; Sharma *et al.*, 1990).

1.2.4. **Disease management**

Prevalent disease management options include crop rotation and manipulation of cultural practices, growing of partially resistant varieties, application of chemical fungicides, and a few biocontrol strategies. Agronomic practices, such as planting less susceptible varieties, early planting, reasonable plant stand, wider plant spacing, weed management, early drainage of rice fields, reduced tillage and proper management of nitrogen fertilizer, can mitigate disease development. However, cultural methods, including crop rotation, have limited benefits for ShB management and may not be effective due to pathogen’s wide host range and its ability to
survive in soil/plant debris for a long time (Lee and Rush, 1983). Resistant varieties could be an effective method to reduce ShB damage and minimize fungicide application, but the development of completely resistant varieties has not been successful due to high genetic variation in the pathogen and the lack of ShB resistant genes in prevalent rice germplasm (Li et al., 1995; Marshall and Rush, 1980; Ou, 1985). Usually, resistance is attributed to tall and late maturing varieties, but desired agronomic features include short stature and early growing rice varieties (Liu et al., 2014). At present, only a limited number of rice varieties, such as Jupiter, Neptune and Tarart, are commercially available with moderate resistance to the ShB pathogen (Sha et al., 2010; Shrestha et al., 2018). Fungicide application offers a significant level of ShB reduction and is a widely preferred strategy by growers. Fungicides with active ingredients azoxystrobin, flutolanil, trifloxystrobin, and propiconazole are currently used for ShB management in the United States, among which azoxystrobin (within the strobilurin group) is the most common and effective in reducing ShB development (Groth and Bond, 2006). However, the use of fungicides is not an economically and ecologically sound option for disease management, its efficacy depends on the timing of application, and the pathogen develops resistance against fungicide within a few years. Therefore, growers are looking for a safer and effective alternative approach for ShB management (Singh et al., 2002). Biological control, thus, has emerged as a possible non-chemical alternative due to its significant eco-friendly and cost-effective nature (Cook, 1993; Gnanamanickam, 2009).

1.3. **Biocontrol of BPB and ShB using bacterial antagonists**

The rich and diverse microbial world provides an endless resource for disease management and growth enhancement of plants. Bioagents have widely been used as biocontrol agents (BCAs), and plant growth promoting rhizobacteria (PGPR) offer safe options to reduce the dependence on agrochemicals. Biocontrol refers to the use of non-pathogenic microbial
antagonists to suppress the activity and growth of pathogen through the production of lytic enzymes, antibiotics, competition for nutrient and space, and induced resistance (Bloemberg and Lugtenberg, 2001; Cook and Baker, 1983). A variety of microbes, such as bacteria, fungi, and viruses, are available in nature and have been studied for their biocontrol activities against major pathogens of rice. Among them, rice-associated bacteria (RABs) isolated from leaves, stem, panicles and rhizosphere are considered to be ideal candidates due to their rapid multiplication, ease of handling and aggressive colonizing ability. Among many potential antagonistic bacteria, members of the genus *Bacillus* and *Pseudomonas* have been successful in biological control of various rice diseases caused by bacterial and fungal pathogens (Cook and Baker, 1983; Gnanamanickam and Krishnamurthy, 1998; Schippers *et al.*, 1987). Bacterial antagonists, mainly *Pseudomonas* and *Bacillus*, are good candidates for biocontrol activities. *Bacillus* spp. are ubiquitous, gram-positive, viable endospore-producing bacteria with good heat and desiccation tolerance, an important feature for field application (Nicholson *et al.*, 2000). The pseudomonads are gram-negative, rod-shaped bacteria with minimum nutrient requirements and better colonizing ability, and are widespread in the rice rhizosphere (Gnanamanickam, 2009).

Several studies have shown the potential of rice associated strains of *Bacillus* and *Pseudomonas* in a significant reduction in infection caused by ShB in rice. Foliar application and seed treatment with antagonistic bacteria *P. fluorescens* reduced the ShB development and increased the crop yield in the field condition (Mew and Rosales, 1992). Similarly, antagonistic bacteria, *P. fluorescens* and *B. subtilis*, suppressed ShB infestation when applied as foliar spray prior to ShB inoculation and were found to colonize rice seeds, stems and roots when applied as a seed treatment (Kanjanamaneesathian, 1994). Application of fermented liquid containing *Bacillus* strain Drt-11 showed reduced sclerotia germination and hyphal growth in an in-vitro
assay and increased plant height and yield in field tests (Chen and Kang, 2006). Also, granules of *B. megaterium* broadcast or sprayed in water were shown to reduce lesion development of ShB in rice in a greenhouse environment (Wiwattanapatapee *et al.*, 2004; Wiwattanapatapee *et al.*, 2007). Three applications of the commercial formulation of *Bacillus subtilis* MBI 600, Integral™ (BASF), as a seed bacterization, seedling dip and foliar spray significantly suppressed the activity of *R. solani* and promoted plant growth and yield under greenhouse and transplanted field conditions (Kotamraju *et al.*, 2012). Foliar application with Serenade Max™ (*B. subtilis* strain QST713, 14.6% a. i., Bayer Crop Science) at the boot stage reduced the damage from ShB and enhanced yield by 15% (Zhou and McClung, 2013). Foliar application of antagonistic *Bacillus amyloliquefaciens* suppressed ShB infection in the *in vitro* leaf detached assay and in the field condition; however, disease suppression was not consistent in a repeated trial (Shrestha *et al.*, 2016). The success of biological control has been inadequate and inconsistent around the world. The efficacy of BCAs requires an understanding of the biological system in which they are applied, and every host-pathogen-antagonist interaction is unique as to time and space (Cook, 1993). In the present context of Louisiana, enough efforts have been placed on the development of ShB resistant rice varieties, but there are few studies on screening of effective BCAs with their formulation and application methods in the rice growing regions of Louisiana.

Scientific research groups have been working on developing effective BCAs for BPB management in rice around the world. Several avirulent strains within the genus *Burkholderia* have been found to control the activity of *B. glumae* (Furuya *et al.*, 2011; Miyagawa and Takaya, 1987). Seed bacterization with avirulent strain N7503 was found to be effective in suppressing the activity of virulent strains of *B. glumae*, a pathogen of bacterial seedling rot of rice (Furuya *et al.*, 2011). Also, foliar spray of rice panicles with avirulent strain of *B. gladioli*, before the
inoculation with *B. glumae*, successfully reduced the incidence of bacterial grain rot in field trials (Miyagawa and Takaya, 1987). Apart from avirulent strains of *Burkholderia* spp., bacteria from the genus *Bacillus* and *Pseudomonas* also have the potential to inhibit the growth of *B. glumae*. Seed treatment with an antagonistic *Pseudomonas* sp. strain suppressed seedling rot when applied as a seed treatment under field environment (Inoue *et al.*, 2001). Likewise, seed treatment followed by foliar application with *Bacillus* strain EXTN-1 reduced the severity of BPB and enhanced yield compared to non-treated control (Zhou *et al.*, 2010). Shrestha *et al.* reported a significant reduction of BPB infection by antagonistic strains of *B. amyloliquefaciens* when applied as a foliar spray on the rice panicles at the heading stage in the field trials in Louisiana (2016). To date, relatively fewer studies have been conducted on screening and biology of potential BCAs for effective BPB management.

1.4. **Rice growth promotion by nitrogen-fixing bacteria**

Nitrogen (N) is an essential nutrient element for cereal crop production, including rice. Plant utilizable N is inadequate in the soil for optimal grain yield, which can be enhanced by adding nitrogen to the soil (Reinhold-Hurek and Hurek, 1998). Rice production heavily relies on inorganic N fertilizers, and their increasing application has been a concern due to its adverse impact on human health and the environment. Due to leaching and runoff problems, plants are unable to utilize commercial chemical fertilizers efficiently, and thus, an alternative source of nitrogen for crop production and growth enhancement is needed (Reddy *et al.*, 2002). Biological nitrogen fixation (BNF) by diazotrophic bacteria, which reduce atmospheric N₂ to plant utilisable ammonium using nitrogenase enzyme complex, can be used as a substitute nitrogen source to supplement inorganic N fertilizers in rice production (Ladha and Reddy, 1995; Lam *et al.*, 1996).
Different microbes, including fungi and bacteria, are associated with rice plants. A class of beneficial bacteria that colonize plant rhizosphere with the ability to promote plant growth by producing growth hormones, inhibiting pathogen growth or enhancing nutrient availability are known as plant growth promoting rhizobacteria (PGPR) (Glick, 2012; Kloeper, 1981; Vessey, 2003). Some of PGPR are diazotrophs, free-living rhizosphere bacteria, that can fix dinitrogen (N₂) to ammonia (NH₃) in non-legumes (Dilworth, 1974). Several diazotrophs have been identified in association with cereal crops, which include bacteria from the genera Azoarcus, Azotobacter, Azospirillum, Burkholderia, Pantoea, Pseudomonas, Rhizobium, Burkholderia and Bacillus (Dobbelaere et al., 2001; Duan et al., 2007; Reinhold-Hurek and Hurek, 1997; Santi et al., 2013). Some rice-associated diazotrophic PGPR can fix atmospheric N₂ as well as promote plant growth via growth promoting mechanisms different from BNF (Dobbelaere et al., 2001).

Inoculation of rice plants with some strains of Burkholderia spp. increased shoot biomass and N contents compared to non-inoculated controls (Divan Baldani et al., 2000). Under greenhouse conditions, seed treatment with a nitrogen-fixing strain of Azospirillum amazonense enhanced yield parameters of rice plants, such as grain dry weight and panicle count, through BNF, which was confirmed by nitrogen accumulation at maturity (Rodrigues et al., 2008). Beneduzi et al. (2008) tested multiple strains of Bacillus spp. and Paenibacillus spp. on rice plants and found that a soil drench with diazotrophic strain Bacillus sp. SVPR30 significantly increased root and shoot length along with dry matter compared to non-treated control plants within 15 and 30 days after sprouting under a greenhouse condition. According to Fent et al. (2006), inoculation of rice plants with the nitrogen-fixing strain, Pantoea agglomerans YS19, enhanced the growth of 12 days-old rice seedlings under the gnotobiotic condition in both N-free and N-supplemented media. Rice seedlings treated with nitrogen-fixing strains of Pantoea sp.
having other PGPR features enhanced the growth of roots and shoot according to the measurement of rice seedlings at 12 days after inoculation (Banik et al., 2016). *Pseudomonas stutzeri* A15 significantly enhanced the growth of rice seedling 6 weeks after inoculation in terms of shoot and dry weight under a greenhouse condition (Pham et al., 2017). In a pot experiment under greenhouse conditions, seed treatment with *Pseudomonas* sp. significantly increased shoot length, biomass and chlorophyll content of rice plants compared to the non-treated control under both low and high N conditions (Wang et al., 2017). Likewise, inoculation of rice seedlings with *P. fluorescens* via the root dipping method enhanced ammonification activity in the soil and also increased root and shoot biomass of rice plants grown in the absence of N (Zhang et al., 2018). According to Greetatorn et al. (2019), the diazotrophic PGPR strain *Bradyrhizobium* sp. SUTN9-2 enhanced dry weight and chlorophyll content of rice plant at an early seedling stage with N-free and NH₄NO₃ under *in vivo* condition when inoculated to germinated seeds. In addition, it was further suggested that SUTN9-2 with its additional ability to produce the plant growth hormone indole acetic acid (IAA) could be helpful for early seedling establishment (Greetatorn et al., 2019).

1.5. Rationale and objectives of the study

Rice (*Oryza sativa* L.), a member of the grass family (Family: Poaceae/Gramineae), is the primary staple food crop for the greater part of the world’s population. It is produced worldwide, with about 90% grown in Asia, and is consumed as one of the primary sources of dietary caloric supply and protein intake in Asia, South America, and Sub-Saharan African regions (Gnanamanickam, 2009). The United States is one of the leading exporter of all types (long, medium and short grain rice) of rice after India, Thailand, Vietnam, and Pakistan (Childs and Skorbiansky, 2018). In the United States, rice is grown in approximately three million acres mainly in six states: Arkansas, California, Louisiana, Missouri, Mississippi, and Texas, with
Louisiana being the 3rd largest rice-producing state (Mcbride et al., 2018). Rice production in Louisiana covers 13% of the overall production of the United States. Rice is one of the main crops grown in Louisiana with a history of cultivation over 300 years (Saichuk et al., 2014).

Growers/scientists are facing a challenge to enhance crop productivity to meet the rising global demand for food with the increasing population. Need for increased crop yield has intensified the agricultural production systems, which in turn, has increased abiotic and biotic pressure on crop plants. Several abiotic stresses such as salinity, drought, flooding, advert temperature, nutrient deficiency/excess as well as biotic stresses, such as pests and diseases, have impaired rice production causing significant quality and yield losses and can be a threat to the US rice industry. Among biotic stresses, rice diseases, such as BPB and ShB, are economically significant in Louisiana and other rice-growing regions, resulting in occasional severe yield and quality losses (Nandakumar et al., 2009). Only partial-resistant varieties for BPB and ShB are commercially available in the United States owing to the lack of effective resistant genes in prevalent rice germplasms (Brooks, 2007). Effective control options are not available for the management of BPB in the United States, whereas ShB management mainly depends on the application of fungicides, which is not a sustainable and eco-friendly option. Also, the application of similar pesticides often leads to the occurrence of resistance in pathogen populations, suggesting a need for developing non-chemical control options. In addition to diseases, nutrients are essential for rice growth. Among the essential plant nutrients, nitrogen is one of the significant elements that limit the growth and productivity of rice plants under most conditions. For optimal crop yield, growers in Louisiana are heavily relying on the use of synthetic fertilizers to meet the N requirement of rice production. Over application of nitrogen has been an increasing concern recently in the context of water management of Louisiana,
(Leonard, 2018) and can hurt crayfish cultivation (Lutz et al., 2011). Agricultural leaching and runoff lead to nutrient pollution (eutrophication) and result in degradation of the quality of ground/surface water due to development of hypoxic zone (low or depleted dissolved oxygen) in water bodies (Bianchi et al., 2008).

The utilization of chemical-based fertilizers and pesticides has a significant impact on the environment, human health and non-target organisms, such as soil microbes, non-target invertebrates and plants, fish, birds, etc., through contamination of soil, water and food resources. Increased dependency on agrochemicals is leading agriculture towards unsustainability, so there is a growing need of eco-friendly alternative inputs to reduce chemical fertilizers and pesticides for managing rice production with minimal negative impacts on human health and environment. One of the sustainable measures for the above problems is the use of beneficial rice-associated bacteria (RABs) as biocontrol agents and/or plant growth-promoting agents.

To date, few RABs (bioagents) have been evaluated for their efficacy in suppressing BPB and ShB in the growing condition of Louisiana, and the method of application has not been thoroughly studied. The occurrence of strobilurin-resistant pathogen population and the unsustainability concern of extensive use of chemical pesticides demand the search of more effective biological control agents and the development of proper methods of application or formulation to enhance their activities. Also, rice associated diazotrophs have not been studied for their growth promotion ability in N-free and N-supplemented growing media in Louisiana. Our findings may pave the way for the development of commercial products that can be directly used by farmers for promoting rice seedling growth (leading to minimizing the use of
commercial fertilizers) and suppressing two major rice diseases (leading to minimizing the use of chemical pesticides).

The objectives of this study were:

1. To evaluate the efficacy of foliar spray with antagonistic *Bacillus* spp. and *Pseudomonas* spp. to suppress ShB and BPB of rice, respectively under field environment.

2. To access the efficacy of seed treatment with antagonistic *Bacillus* spp. to reduce ShB development under greenhouse environment.

3. To isolate and screen nitrogen-fixing rice rhizobacteria and test their potential to promote the growth of rice seedlings in the presence or absence of nitrogen.

Chapter 2 will focus on the evaluation of antagonistic bacteria for their biocontrol activities against ShB and BPB as foliar and/or seed treatment under different conditions and will cover objectives 1 and 2, whereas chapter 3 will focus on the evaluation of nitrogen-fixing bacteria for their growth-promoting ability on rice at the seedling stage.
CHAPTER II. EVALUATION OF *Bacillus* AND *Pseudomonas* Strains for Bio-control Activities on Sheath Blight and Bacterial Panicle Blight of Rice

2.1. Introduction

Bacterial panicle blight (BPB), primarily caused by the seed-borne bacterial pathogen *Burkholderia glumae*, is one of the significant economic rice diseases in the Southern United States including Louisiana. High night temperatures followed by frequent rainfall favor disease development, which can cause epidemic resulting in losses up to 40% (Cha *et al.*, 2001; Shahjahan *et al.*, 2000). Typical symptoms of BPB include discolored panicles, brown linear lesions on the sheath and upright infected panicles (due to failure of grain formation) under disease conducive environmental conditions (Nandakumar *et al.*, 2009). Few effective methods have been reported to manage BPB, although hot water treatment of seed and commercial varieties, such as Jupiter, with partial resistance are recommended to reduce the disease (Ham and Groth, 2011). Currently, stable and effective chemicals have not been developed for the management of BPB (Ham *et al.*, 2011). Some copper based products are available, but they are not very effective and are sometimes phytotoxic to rice plants (Xu *et al.*, 2006).

Sheath blight disease (ShB), caused by the soil-borne fungal pathogen *Rhizoctonia solani*, is one of the most important diseases of rice in the world capable of causing severe yield losses up to 50% in rice (Lee and Rush, 1983). The introduction of high yielding varieties combined with high nitrogen fertilizer application promoted ShB as a significant disease in Louisiana and other southern rice-growing states in the United States (Groth and Lee, 2003). Typical symptoms begin with water-soaked lesions on the base of the leaf sheath near water line. The lesion then spreads to upper sheaths and leaves, including the flag leaf, under favorable environmental conditions (Lee and Rush, 1983). Due to the unavailability of high yielding
varieties with high resistance against ShB (Li et al., 1995; Marshall and Rush, 1980; Ou, 1985), strobilurin-based fungicides, such as azoxystrobin, provide the main method for ShB management. However, the application of fungicides is costly and not always effective (Groth, 2005), and poses potential health, safety and environmental risks (Willocquet et al., 2000). In addition, fungal pathogen populations may develop resistance against fungicides. In 2010-11, strobilurin-resistant isolates of _R. solani_ were reported in various rice fields in Acadia Parish, Louisiana (Olaya et al., 2012), and resistance is predicted to continuously develop and spread into new areas in Louisiana.

Due to the lack of effective management practices along with human and environmental health issues and the emerging problem of pesticide resistance resulting from long-term application of fungicides, alternative management options for ShB are needed. Biological control agents (BCAs) have been considered as a promising, safe option, and offer low risk of resistance development due to their complex mechanisms for action. Plant-associated microorganisms, isolated from a specific crop under a particular environment, are better adapted to the plant tissues of interest under similar environmental conditions and can therefore provide better biocontrol activities against plant diseases (Cook, 1993). Rice-associated bacteria (RAB), from the genera _Bacillus_ and _Pseudomonas_, have shown antagonistic activities against major bacterial and fungal pathogens of rice (Gnanamanickam and Krishnamurthy, 1998; Schippers et al., 1987). The ubiquitous nature and heat and desiccation tolerance ability of _Bacillus_ and minimal nutrient requirements and high colonization potential of _Pseudomonas_ strains make them good candidates for biocontrol of various diseases (Gnanamanickam, 2009; Nicholson et al., 2000). Different strains of BCAs have shown potential to manage ShB and BPB around the world (Gnanamanickam, 2009); however, few potential BCAs have been studied in rice growing
conditions of Louisiana and isolates with potential for the effective control and for practical implementation in commercial agriculture in Louisiana have not yet been identified.

The objectives of this study were as follows:

a) To evaluate antagonistic *Bacillus* spp. and *Pseudomonas* spp. as a foliar spray for suppression of ShB and BPB of rice, respectively, under field conditions.

b) To evaluate antagonistic *Bacillus* spp. as a seed treatment for suppression of ShB under greenhouse conditions.

2.2. Materials and Methods

2.2.1. Invitro screening and identification of antagonistic bacteria

RABs previously isolated from rice plants (Supplementary Table 1.1) at the Rice Research Station at Crowley, Louisiana, were screened for their antagonistic activities against the pathogens by growth-inhibition plate assays as previously described (Shrestha *et al.*, 2016). For antifungal activity, a mycelial plug of *R. solani* (5-mm-diameter) was placed on the middle of a potato dextrose agar (PDA) plate. Each bacterial strain was grown in Luria broth (LB) (10 g tryptone, 10 g NaCl and 5 g yeast extract per L), and 5 µL of each culture (OD$_{600} = 0.1$) was spotted three places on the PDA plates around the center. The plates were incubated at 28±2 °C, and antifungal activity (development of inhibition zone) was observed after 72 h. Similarly, 100 µL of overnight grown *B. glumae* at OD$_{600} = 0.1$ was spread with a glass inoculation stick on Luria broth agar (9 gm agar added to L broth). Five µL of each bacterial culture was dropped on three spots on LB agar plates followed by incubation of plates at 28±2 °C. The plates were examined for antibacterial activity (development of inhibition zone) and zones of inhibition were measured after 48 h.

Bacteria showing antagonistic activities were previously identified (Supplementary Table 1.1). For the identification, genomic DNA of bacterial isolates was extracted by using Sigma
Aldrich’s GenElute Bacterial Genomic DNA Kit. The 16S ribosomal DNA sequence was amplified for identification of bacterial isolates using the following primers: fD1 (50-AGAGTTT-GATCCTGGCTCAG-30) and rP2 (30-ACGGCTACCTTGT-TACGACTT-50) (Weisburg et al., 1991). For identification, BLAST (Basic Local Alignment Search Tool) from NCBI Gene Bank (www.ncbi.nlm.nih.gov) was used for homology search to find the closest species based on the 16S rDNA sequence data (Supplementary Table 1.1).

2.2.2. Field experiments for the management of BPB and ShB

The experiments were conducted at the Rice Research Station at Crowley, Louisiana, with treatments organized in a randomized block design with four blocks (Figure 2.1). Bengal (an early maturing, semi-dwarf, medium-grain variety, susceptible to BPB) was grown for the BPB experiment, whereas CL–111 (a very-early short stature, long grain Clearfield rice variety, susceptible to ShB) was grown for the ShB management experiment. The seeding rate for both varieties was 112.08 kg/ha. Soil features included Crowley silt loam type, pH 6.0, clay 12%, silt 71%, sand 17%, cation exchange capacity (CEC) 9.4 kg\(\text{ha}^{-1}\). The size of each plot was 1.2 m by 4.8 m (= ~ 5.8 m\(^2\)). The gap between two blocks was 1 m and between two plots was 0.5 m.

Rice plants were artificially inoculated with *R. solani* at the tillering stage, while *B. glumae* was inoculated at the early heading stage. *B. glumae* 336gr-1 was cultured overnight on LB agar at 37 °C and resuspended in the buffer solution (10mM MgCl\(_2\)) adjusting the final concentration to ca. 1*10\(^8\) CFU/ml. The bacterial solution was sprayed on the rice panicles. Whereas, inoculum for *R. solani* was prepared using rice husks/grain mixture with 1:2 ratio following the method previously developed in our lab (Shrestha et al., 2016). The inoculum was scattered by hand to the lower portion of plants. Selected antagonistic bacteria (Table 2.1) were applied via spraying on rice sheaths (for ShB) or panicles (for BPB) of plants 24 h after
**Figure 2.1.** A field map showing positions of treatments and blocks.

For BPB, treatments were T1 = Non-inoculated, non-treated control (buffer treated), T2 = Non-treated, inoculated control, T3 = Quadris (0.84 kg/ha), T4 = RAB14 R, T5 = RRB985, T6 = REB711, T7 = RAB14 R/ with culture, T8 = RRB985/ with culture and T9 = REB711/ with culture. For BPB, treatments were T1 = Non-inoculated control (buffer treated), T2 = Inoculated control, T3 = Kocide (3.36 kg/ha), T4 = RPBNT5, T5 = RRB1044, T6 = RRB1047, T7 = RPBNT5/ with culture, T8 = RRB1044/ with culture and T9 = RRB1047/ with culture. RRB - Rice Rhizosphere Bacteria, REB – Rice Endophytic Bacteria, RPB – Rice Panicle Bacteria.
inoculation with the agar plates (solid media) and in LB broth (liquid culture). The bacterial treatments were prepared in two different growing media (agar plates and liquid broth) to compare the effect of growing media. The bacterial growth was mixed with buffer solution (10 mM MgCl₂ – prepared using sterile water), adjusting the final concentration to ca. 1*10⁸ CFU/ml. Silwet-77 (0.05%) was added as a surfactant. Each bacterial suspension was sprayed on the plants until runoff (~500ml/plot). The plots sprayed with buffer solution 10 mM MgCl₂ were used as non-treated, non-inoculated control. The azoxystrobin fungicide, Quadris (0.84 kg/ha), was included as a chemical control for ShB experiment.

For evaluation of ShB, ten (2017) and 15 (2018) plant samples were randomly selected from each plot 4 weeks after inoculation of pathogen, and the final disease severity was scored based on the scale of 0 – 9 based on the presence of lesion relative to the plant height (Figure 2.2) (IRRI, 1996). For BPB, a single plot was divided into six parts and individual parts were observed for symptomatic panicles, making six ratings per plot. The disease severity of the rice panicles was scored 2 weeks after inoculation based on the percentage of discolored area with the scales ranging from 0 to 9 as follows: 0 = no infection, 1 = 1-20 % of panicles, %, 3 = 21-40 %, 5 = 41-60 %, 7 = 61-80 %, 9 = > 80 %.

**Table 2.1.** Selected antagonistic bacteria for field experiments.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Antagonism against</th>
<th>Species with highest homology match</th>
<th>% Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPBNT5</td>
<td><em>B. glumae</em> (BPB)</td>
<td><em>Pseudomonas parafulva</em></td>
<td>97.1</td>
</tr>
<tr>
<td>RRB1044</td>
<td><em>B. glumae</em> (BPB)</td>
<td><em>Pseudomonas plecoglossicida</em></td>
<td>96.5</td>
</tr>
<tr>
<td>RRB1047</td>
<td><em>B. glumae</em> (BPB)</td>
<td><em>Pseudomonas putida</em></td>
<td>95.7</td>
</tr>
<tr>
<td>REB711</td>
<td><em>R. solani</em> (ShB)</td>
<td><em>Bacillus amyloliquefaciens</em></td>
<td>98.5</td>
</tr>
<tr>
<td>RRB985</td>
<td><em>R. solani</em> (ShB)</td>
<td><em>Bacillus subtilis</em></td>
<td>97.5</td>
</tr>
<tr>
<td>RAB14R</td>
<td><em>R. solani</em> (ShB)</td>
<td><em>Bacillus amyloliquefaciens</em></td>
<td>97.3</td>
</tr>
</tbody>
</table>

2.2.3. **Greenhouse experiment for evaluation of Bacillus spp. for ShB management**

Rice seeds (cv. CL–111, 2018) were surface sterilized with 5% sodium hypochlorite solution (available chlorine 5%) adjusted to pH 7.0 (1 M hydrochloric acid/1 M sodium
Figure 2.2. Disease score of ShB based on the presence of lesion relative to plant height (PH).

Assigned ShB scores were: 0 = No lesions observed, 1 = lesion confined to lower 20 % of plant height, 3 = lesion confined to 20–30 % of plant height, 5 = lesion confined to 31–45 % of plant height, 7 = 46–65 %, 9 = lesion confined to > 65 % of plant height (IRRI, 1996). The arrows in the picture indicate the area of ShB lesions on the sheaths and leaves.

hydroxide) for 15 minutes followed by treatment with 0.1 M HCl for 10 min, and were rinsed with sterile water eight times (Abdul-Baki, 1974; Abdul-Baki and Moore, 1979; Footitt et al., 1995). Antagonistic bacterial isolates grown on LB agar were suspended in a buffer solution (10 mM MgCl₂) with the final concentration adjusted to ca. 2*10⁹ CFU/ml. After surface-sterilization, rice seeds were soaked in each bacterial suspension with 2% carboxymethyl cellulose (CMC) added as a sticker in a 150 ml flask and incubated on a shaker at 180 rpm for 1 h at 30±2°C. After incubation, the excess bacterial suspension was discarded, and the treated
seeds were placed in a laminar flow hood overnight (~16 h). Seeds treated with sterile buffer were used as a control in this study.

Each experiment was conducted with treatment groups arranged in a completely randomized design with five replications for each treatment group: three Bacillus strains + two controls (inoculated and non-inoculated). Each replication included three seedlings. Ten rice seeds were directly sown in a 10-cm-square pot (639 cm³ of sterile soil/sand mixture in a 3:1 ratio) and thinned to three uniform rice seedlings at 10 days after sowing. Inoculation of R. solani and rating of disease severity were carried out following the procedure explained by Park et al. (Park et al., 2008) as shown in Figure 2.3. At the tillering stage of rice plants (typically 8-weeks), leaf sheaths were inoculated by attaching a PDA block (diameter 0.5 cm) containing 3-day old mycelia of R. solani to each rice sheath, which were then covered with the aluminum foil. Non-inoculated control represents the plants inoculated with PDA blocks free of R. solani mycelium, whereas non-treated plants represent no inoculation and no bacterization of seeds. The foils on the leaf sheaths were discarded after the appearance of lesion (usually 3 days), and lesion length and disease severity were recorded 7 days after inoculation.

2.2.4. Statistical analysis

All the experiments were repeated once to confirm the treatment effects. Data were analyzed using a proc mixed linear model (SAS Institute, Inc., Cary, NC). Proc mixed allows modeling of random and mixed effect data, handling of unbalanced data and simplifying analysis related to repeated measures, data with heterogeneous variances and autocorrelated observations. The results were expressed as mean ± standard deviation (SD) for each treatment group and the significance was evaluated at p < 0.05 (F-test) for all experiments. Fisher’s protected least significance difference (LSD) was used to determine any significant differences among treatment
groups. Interaction of treatment and environment was significant, so data from two independent trials are presented.

![Figure 2.3. Disease ratings of ShB under the greenhouse condition. Assigned ShB scores were: 0 = No symptomatic lesion, 1 = presence of water-soaked lesions, 2 = presence of necrotic lesions, 3 = < 50% necrosis on the leaf cross section, 4 = > 50% necrosis on the leaf cross section, and 5 = necrosis across the entire leaf section or death of leaf (Park et al., 2008).](image)

2.3. Results

2.3.1. In vitro screening of potential antagonistic bacteria

All the isolates were screened for antagonistic activity against both rice pathogens, *B. glumae* and *R. solani*, in a growth inhibition assay. Several antagonistic bacterial isolates inhibited the growth of rice pathogens showing a clear inhibition zone (Figure 2.4). Out of several candidate strains, three strains each of *Pseudomonas* spp. (RPB NT5 (*P. parafulva*), RRB 1044 (*P. plecoglossicida*) and RRB 1047 (*P. putida*)) and *Bacillus* spp. (REB 711 (*B. amyloliquefaciens*), RRB 985 (*B. subtilis*) and RAB 14R (*B. amyloliquefaciens*)) were selected on the basis of their antagonism for further evaluation in the field against BPB and ShB,
respectively (Figure 2.4). Thirty-five rice-associated bacterial isolates (Supplementary Table 1.1) were subjected to homology search to find the closest species based on the 16S rDNA sequence data.

![Image](image_url)

**Figure 2.4.** In vitro screening of antagonistic bacteria by growth-inhibition plate assays. Antagonistic activity of *Pseudomonas* spp. against *Burkholderia glumae* on LB agar (A) and *Bacillus* spp. against *Rhizoctonia solani* on PDA plates (B). *Pseudomonas* strains RPBNT5 (*P. parafulva*), RRB1044 (*P. plecoglossicida*), RRB 1047 (*P. putida*) were selected for field trials against BPB, whereas *Bacillus* strains REB711 (*B. amyloliquefaciens*), RRB985 (*B. subtilis*), RAB14R (*B. amyloliquefaciens*) strains were selected for field trials for ShB management.

### 2.3.2. Field experiments for the management of BPB and ShB

Two trials were carried out at Crowley, Louisiana, in 2017 and 2018 to evaluate the performance of three selected antagonistic rice-associated bacteria for BPB and ShB management, respectively, under field conditions. Figure 2.5 represents the biocontrol activities of selected antagonistic *Pseudomonas* strains against the disease development of BPB. The severity of BPB symptoms on the rice panicles in all the treated and non-treated plots was very low in both seasons with an average disease severity rating ranging from 1 – 3 (Figure 2.5 A and (image_url)
B). Therefore, the biocontrol efficacy of the *Pseudomonas* strains could not be determined meaningfully due to the low disease pressure in both field trials of 2017 and 2018. However, copper fungicide Kocide 3000 @ 3.36kg/ha used in the field trials produced severe damage to rice panicles by itself, indicating its phytotoxicity problem in applications for the control of BPB (Figure 2.6). Even the lowest concentration of Kocide 3000 (1.1 kg/ha) had phytotoxic effects on the rice panicles (data not shown).

Considerable development of ShB lesions occurred in the 2017 and 2018 trials, so it was possible to determine treatment effects (Figure 2.7). In 2017, all three strains suppressed ShB development compared to the non-treated, inoculated control with the disease reduction ranging from 18 – 34 % (Figure 2.7 A). *Bacillus* strains REB 711 and RRB 985 and RAB14R (/WC) reduced ShB development by 30 % and 25 – 33 %, respectively, which was similar to 47% ShB development reduction by azoxystrobin (Quadris as chemical control). However, in 2018, only REB711 significantly reduced ShB development compared to the non-treated control (Figure 2.7 B). In the 2018 field trial, only *Bacillus* strain REB 711 consistently reduced the ShB damage (23 – 30 %) compared to the non-treated control, and it was less effective than the fungicide azoxystrobin (Quadris – chemical control). The fungicide azoxystrobin completely suppressed ShB under lower disease pressure in 2018 compared to 2017 trial, when moderate natural infection was observed in the non-inoculated plots (Figure 2.7). The form of culture media (solid agar plate vs. liquid broth) to grow the bacteria for inoculum did not make any significant difference in the efficacy to manage disease (Figure 2.7), although the bacteria grew better on solid agar plates (higher bacterial cells - data not shown). In both test years, REB711 suppressed disease development under field conditions when it was foliar-sprayed on the rice plants.
Figure 2.5. Evaluation of three *Pseudomonas* strains to suppress BPB under field conditions in 2017 (A) and 2018 (B). Bacterial cells of antagonistic bacteria, initially grown on either solid LB agar plate or liquid broth (whole broth culture, WC), were sprayed on the rice panicles to evaluate ShB suppression and compared to Kocide 3000 applied as a chemical control and two controls: non-inoculated, non-treated plants and inoculated, non-treated plants. Error bar indicates standard deviation for each treatment. Mean values with different letters (Fisher’s LSD) were significantly different (P<0.05).
2.3.3. Greenhouse experiments for the evaluation of Bacillus spp. for ShB management

Based on the results from the field experiments, the efficacy of the three Bacillus strains for ShB management was further evaluated by seed treatment under greenhouse conditions. Non-inoculated control plants did not develop disease symptoms, whereas inoculated, non-treated plants developed significant levels of ShB symptoms on leaf sheaths and blades in both trials. In the first trial, seed treatment with the strain REB711 significantly reduced disease development in plants in terms of disease rating (by 52%) and lesion length (by 63%) in comparison with the non-treated control (Figure 2.8 A and B). REB711 was significantly different from RRB985 and RAB14R in the reduction of ShB symptoms. RAB14R also showed significant reduction in disease ratings compared to non-treated, inoculated control. Similar disease reduction was recorded for REB711-treated plants in the second trial, where the disease severity rating was reduced by 44% and lesion length by 49% (Figure 2.8 B and D). RRB985 and RAB14R showed less disease inhibition compared to REB711, and RRB reduced ShB compared to the inoculated control (Figure 2.8).

Figure 2.6. Severe damage caused by copper fungicide (Kocide 3000) in rice panicles. Phytotoxicity symptoms on plots treated with Kocide in the field (A). Phytotoxicity symptoms on rice panicles (B).
Figure 2.7. Evaluation of three *Bacillus* strains to suppress ShB under field conditions in 2017 and 2018. Bacterial cells of antagonistic bacteria, initially grown on either solid LB agar plate or liquid broth (whole culture, WC), were sprayed on the rice panicles to evaluate ShB suppression and compared to azoxystrobin applied as a chemical control and two controls: non-inoculated, non-treated plants and inoculated, non-treated plants. Error bar indicates standard deviation for each treatment. Mean values with different letters (Fisher’s LSD) were significantly different (P≤0.05).
Figure 2.6. Evaluation of three *Bacillus* strains to suppress ShB under greenhouse conditions. Disease was assessed as lesion length and a disease severity rating recorded 7 days after inoculation with *R. solani*. Lesion length for trial 1 (A), and trial 2 (C). ShB score for trial 1 (B) and trial 2 (D). Assigned disease ratings were: 0 = no lesion, 1 = presence of water-soaked lesions, 2 = presence of necrotic lesions, 3 = < 50% necrosis on the leaf cross section, 4 = > 50% necrosis on the leaf cross section, and 5 = necrosis across the entire leaf section or death of leaf. Error bar indicates standard deviation for each treatment. Mean values with different letters (Fisher’s LSD) were significantly different (P≤0.05).

2.4. Discussion

In this study, the antagonistic potential of locally obtained RABs isolates were first evaluated *in vitro* against the bacterial rice pathogen *B. glumae* and the fungal rice pathogen *R. solani*. The rhizosphere soil and plant parts of rice plant are good sources for isolates of the
genera *Pseudomonas* and *Bacillus*, and these isolates have been reported to inhibit the growth and activity of several bacterial and fungal pathogens through several mechanisms (Cook and Baker, 1983; Mercado-Blanco, 2015; Shafi et al., 2017). Three antagonistic *Pseudomonas* spp. and *Bacillus* spp. isolated in Louisiana were chosen to further evaluate as potential biological control candidates for the management of BPB and ShB, respectively, under Louisiana field conditions.

The frequency of BPB symptomatic plants and disease severity on rice panicles were low in both field trials suggesting that the environmental conditions were not favorable for disease development in those seasons. Therefore, the treatment effects of foliar spray of *Pseudomonas* strains in managing BPB could not be determined meaningfully in both field trials. The environmental conditions responsible for low BPB severity are unclear. Extended high night temperatures and high humidity followed by frequent rainfall are known to be the determining factors for BPB epidemics (Cha et al., 2001), but the years 2017 and 2018 were similar to other years in those environmental conditions. Similar low disease pressure problems occurred in other plots at the Rice Research Station, where rice plants were also inoculated by another research group for different studies. It might be possible that inoculation of *B. glumae* in the early morning caused prolonged exposure of the pathogen to UV rays and, consequently, reduced the efficacy of the inoculation (Karki and Ham, 2014; Sagripanti et al., 2009). The disease pressure of BPB was low in successive years of field trials with unclear reasons. As a result, the need remains for the development of better and more viable biocontrol agents under the current conditions of Louisiana.

In addition, plots treated with copper compound Kocide 3000 at the rate of 3.36 kg/ha caused considerable damage on the rice panicles in one season, indicating the phytotoxic effect
of copper to the rice plants. The recommended application rate of Kocide 3000 for field crops ranges from 0.5 – 3.5 kg/ha for one application (DuPont™). Even the lower concentration of 1.1 kg/ha was toxic to the rice plants (data not shown). Despite their antimicrobial properties, copper products have been reported to cause toxic effects to many crops, including rice, corn, peanut and soybean (Borkert et al., 1998).

The ability to form endospores, successful colonizing ability, and tolerance of adverse environmental conditions make Bacillus a promising BCA candidate to protect against ShB and several other crop diseases (Errington, 2003). In this study, we evaluated the efficacy of three antagonistic Bacillus isolates to reduce ShB severity. Results of in vitro, field and greenhouse experiments indicated REB711 as an effective candidate for inhibiting R. solani. The foliar application of REB711 significantly reduced ShB symptoms in comparison to the non-treated, inoculated control under field conditions in two seasons. Previous studies also have reported the success of Bacillus strains in inhibiting ShB development in rice (Kotamraju et al., 2012; Shrestha et al., 2016; Zhou and McClung, 2013).

The biocontrol efficacy of REB711 was further confirmed when seed treatment with REB711 showed higher inhibition of lesion development on leaf sheaths and blades of rice plants under greenhouse conditions. The disease reduction by the seed treatment of REB711 under greenhouse conditions indicated successful colonization of the Bacillus strain in rice plants. The reduction in disease development was further reinforced by previous studies where seed treatment increased the colonization activities on different plant parts (Correa et al., 2009; Kanjanamaneeesathian, 1994). Inhibition of the fungus R. solani by REB711 may involve a single or multiple mechanisms, including competition for space and nutrients, production of antimicrobial compounds (peptides, lipopeptides, antibiotics and enzymes), and induction of
systemic resistance (Compant et al., 2005). Studies have reported the suppression of different fungal pathogens through the production of secondary metabolites, such as surfactin, fengycin, bacillomycin, bacteriocins, bacilysin, and difficidin, produced by Bacillus spp. (Chen et al., 2009; Koumoutsi et al., 2004; Stein, 2005). A mechanism of antibiosis might be highlighted by biochemical evidence. The other two strains were found to be inconsistent in their performance against ShB development. The inconsistency and reduction of performance of these Bacillus spp. strains might be caused by the environmental factors during the growing season or by long-term storage conditions. The efficacy of antagonists to suppress ShB depends on the ability of antagonists to survive and remain active until the maturity of the rice plant (Mew and Rosales, 1992). The efficacy of REB711 was less compared to the disease reduction provided by azoxystrobin fungicide; however, the use of Bacillus strain REB711 as a BCA could mitigate the risks of health and environmental issues and pesticide resistance.
CHAPTER III. ISOLATION AND SCREENING OF RICE RHIZOBACTERIA FOR DIAZOTROPHIC POTENTIAL AND THEIR EFFECT ON THE GROWTH OF RICE SEEDLINGS

3.1. Introduction

Nitrogen (N), an important component for plant proteins and nucleic acid, is one of the significant growth-limiting nutrient factors for rice production. However, availability of plant utilizable N in the soil is insufficient for optimum crop yield (Follett and Hatfield, 2001), which requires the external application of N-based commercial fertilizers in the field (Reinhold-Hurek and Hurek, 1998). Dependence on chemically synthesized N fertilizers for rice production is increasing in Louisiana, leading the rice cultural system towards more unsustainable. High input of synthetic fertilizers, with leaching and run-off problems, poses a great threat to water resource by degrading water quality (Bianchi et al., 2008; Leonard, 2018) and affecting crawfish cultivation in Louisiana (Lutz et al., 2011; Nielsen et al., 1999). High N-demanding nature of rice production, as well as economic and environmental issues of chemical fertilizers, urges farmers and investigators to search for economically and environmentally sound alternative options to reduce chemical fertilizer without compromising crop yield (Reddy et al., 2002).

A possible sustainable alternative to inorganic fertilizers can be the use of biological nitrogen fixation (BNF) by N₂ fixers to supplement N to plants (Ladha et al., 1997). Nitrogen-fixing bacteria (also called diazotrophs) can convert atmospheric N₂ into ammonia (utilizable forms) through BNF using the nitrogenase enzyme complex (Kim and Rees, 1994). Several bacterial strains from the genus Azotobacter (Dobereiner, 1961), Azoarcus, Azospirillum (Rodrigues et al., 2008), Pseudomonas (Qui et al., 1981), Klebsiella, Enterobacter (Bally et al., 1983; Ladha et al., 1983), Flavobacterium (Bally et al., 1983), and Herbaspirillum (Baldani et al., 1986) have been isolated from rice rhizosphere as nitrogen-fixing bacteria. Besides nitrogen
fixation, diazotrophs have also been reported to promote plant growth as plant growth promoting rhizobacteria (PGPR) by producing phytohormones, enhancing nutrient availability and increasing tolerance to several abiotic and biotic stresses (Dobbelaere et al., 2001; Kandel et al., 2015).

Research on PGPRs and diazotrophs has been going on for decades, and there are several reports of new diazotrophic bacteria and their use in non-legume crops (Elbeltagy et al., 2001; Gyaneshwar et al., 2001); however, their growth promotion potential has not been thoroughly investigated (Bally and Elmerich, 2007). Moreover, a promising strain can be potent only if it is effective in the given field condition, active to fix N₂ along with the presence of nitrogenase enzyme (James, 2000), and able to supply adequate N for plant growth. To the best of our knowledge, rice-associated diazotrophs isolated from rice fields have not been investigated for their growth promotion ability in N-free and N-supplemented growing medium in rice in Louisiana.

In the present study, 186 bacteria were isolated from the rhizosphere of rice, screened for nitrogen-fixing abilities and further evaluated for their ability to promote the rice growth at the initial seedling stage in the presence and absence of N nutrient. In addition, selected bacterial isolates were labeled with GFP to observe their colonization potential in rice plants.

3.2. Materials and methods

3.2.1 Isolation of rhizosphere bacteria

Rhizosphere soil samples were taken by uprooting the roots of randomly selected rice plants from the rice field in the H. Rouse Caffey Rice Research Station (Crowley, Louisiana). Ten grams of rhizosphere soil was suspended in 90 mL of sterile buffer solution (10 mM MgCl₂) using a rotary shaker at 150 rpm for 30 – 90 minutes. The sample was serially diluted and 0.1 mL aliquots of $10^{-3} – 10^{-5}$ dilution were spread over Luria-Bertani (LB) (10 g tryptone, 10 g
NaCl and 5 g yeast extract per L) agar plates supplemented with cycloheximide (Cm), 40 μgL⁻¹ (Bertani, 1951) and incubated at 30±2 °C. Morphologically distinct bacterial isolates were sub-cultured and stored as a glycerol stock (30% glycerol v/v) in -80 °C.

3.2.2. Screening of nitrogen-fixing bacteria

Isolated rhizobacteria were initially screened for nitrogen-fixing ability by using semi-solid nitrogen (N)-free medium (NFb) containing bromothymol blue (BTB) as an indicator. The components of Dobereiner’s nitrogen free semi-solid media were: malic acid (5g/L) as a carbon source, KOH (4 g/L), K₂HPO₄ (0.5g/L), FeSO₄.7H₂O (0.05g/L), MnSO₄.7H₂O (0.01 g/L), MgSO₄.7H₂O (0.01 g/L), NaCl (0.02 g/L), CaCl₂ (0.01 g/L), Na₂MoO₄ (0.002 g/L), D/W (1000mL), bromothymol blue (BTB) 0.5% alcoholic solution (2 mL) and agar (1.75 g/L) (Day and Döbereiner, 1976). pH was adjusted to 6.6 to 6.8 before autoclaving at 121 °C for 15 min. For solid NFb medium, agar (2%) and yeast extract (0.005 %) was added to semi-solid NFb medium (Day and Döbereiner, 1976). The semi-solid condition aids bacteria in producing nitrogenase complex and initiating N-fixation by creating a microaerophilic environment (Cassán et al., 2015).

First, the strains were inoculated on a semi-solid medium. A change in color was observed after 3–10 days of incubation at 30±2 °C indicating the nitrogen-fixing potential of the isolates. The color of media changes from green to blue due to the change in pH to basic. Increase in the pH value is caused by reduction of atmospheric N₂ to NH₃ (ammonia) by bacteria having the nitrogenase complex (combined of two proteins - an iron protein and a molybdenum-iron protein) (Döbereiner et al., 1972). Based on the intensity of color change, the five best isolates were selected for testing of their ability to promote the growth of rice seedlings along with *Sinorhizobium meliloti* (Dr. Doerrler’s lab) as a control (Scupham et al., 1996).
Also, the selected isolates were tested for ammonia production. Freshly grown bacterial cultures were inoculated into peptone water (1g peptone, 5g sodium hydroxide, 100 mL ammonia-free water) and incubated for 7 days at 30±2 °C. The bacterial culture was centrifuged, and supernatant was collected using pipette. The supernatant was added with 1 mL Nessler’s reagent (10g mercuric chloride, 7g potassium iodide, 16g sodium hydroxide added to 100 mL ammonia-free water and adjusted to pH 13.2) in 1:1 ratio. The final volume was adjusted to 10 mL by adding ammonia-free double-distilled water. Ammonia production was indicated by the development of a yellow to brown color, and its intensity was estimated using a spectrophotometer at 450 nm (Demutskaya and Kalinichenko, 2010).

3.2.3. 

**Seed treatment with selected nitrogen-fixing bacteria**

Rice seeds (Bengal variety, 2018) were provided by the Rice Research Station (Crowley, Louisiana). The damaged seeds were discarded by the water floating method, and the rest of the seeds were collected and air-dried. The air-dried seeds were surface-sterilized using 5% sodium hypochlorite adjusted to pH 7.0 (1 M hydrochloric acid/1M sodium hydroxide) for 15 min followed by treating them with 0.1 M HCl for 10 min and rinsing with sterile water eight times (Abdul-Baki, 1974; Abdul-Baki and Moore, 1979; Footitt et al., 1995). After a 100 μL final wash, seed were plated on LB agar plates and incubating at 30±2 °C for 48 h to confirm the effectiveness of sterilization procedure.

The bacterial cells from overnight grown bacteria (on LB agar) were collected and mixed with a buffer solution (10 mM MgCl₂) and adjusted to a final concentration of ca.2*10⁹ CFU/ml. The surface sterilized seeds were treated with each bacterial suspension (2% CMC added as a sticker) in a 150 ml flask and placed in an incubator for 1 h at 30±2 °C. After incubation, the excess bacterial suspension was discarded, and seeds treated with bacterial isolates were placed
in a laminar flow overnight (nearly 16 h) for drying. Seeds treated with sterile buffer were used as a control.

3.2.4. Growth chamber and greenhouse experiments

Three uniformly germinated seeds (2-3 days) free of visible signs of microbial contamination were transferred to each 50 mL falcon conical centrifuge tubes with 35 g of autoclaved sand and then were thinned to one seedling/tube after 7 days. The setup was in a growth chamber with 12 h photoperiod, 30±2 °C day temperature, 25 °C night temperature, and relative humidity of 65%. Initially, the tubes were supplemented with Yoshida’s nutrient solution (with N and without N) at pH 5.5 at the recommended rate (90 kg/ha) for rice seedling stage (Yoshida et al., 1976). The source of N was water soluble NH₄NO₃ at 40 ppm. When needed, tubes were watered with distilled deionized water at field capacity. The rice seedlings were collected after 21 days. Seedlings were evaluated for biological nitrogen fixation (Soil-Plant Analysis Development (SPAD) readings for chlorophyll content) and growth (plant height and dry shoot weight). Three trials with five replications were conducted in the growth chamber. Later, all replications were combined to determine the treatment effects.

A similar experiment was set up under the greenhouse condition. Ten treated seeds were directly sown in 10 cm square pots (639 cm³ sand) and thinned to five uniform rice seedlings at 10 days after sowing. Measurements of the variables were taken at 30 days of seedling age. The greenhouse experiment was repeated once to confirm the treatment effects.

The experiments were carried out in a completely randomized design with the factorial setup as follows:

Factor 1: bacterial isolates (five) + control (S. meliloti) + control (buffer 10mM MgCl₂)
Factor 2: fertilizer (Yoshida nutrient solution) with and without nitrogen (NH₄NO₃)

Growth chamber: 5 replications * 3 trials (15 replications)
Greenhouse: 5 pots with 5 seedlings/pot * 2 trials

Plant height was measured from the base of the seedling to the tip of the longest leaf. Chlorophyll content in a leaf was measured non-destructively using a SPAD 502 Plus chlorophyll meter (Manufactured by Spectrum Technologies). Leaf transmittance measured by SPAD optical meter estimates chlorophyll concentration in leaves. A high correlation has been reported between N status and leaf chlorophyll content (Evans, 1983), so leaf N status can be determined by SPAD values. Higher SPAD readings imply higher chlorophyll and N content in a leaf. The average of five SPAD readings was taken from the second leaf from the top (Ata-Ul-Karim et al., 2016). The plant samples were oven dried at 70 °C for 48 h to obtain dry weight.

Statistical analysis was carried out using Proc Mixed, a mixed linear model from the SAS program (Ver. 9.4). All data were presented as mean ± standard error (SE) for each treatment and analyzed using an F-test of significance (p < 0.05). Protected Fisher’s LSD was performed to determine any significant differences among treatments. Interaction between treatments and experiment were checked before combining all test into one analysis.

3.2.5. Identification of bacterial isolates

The selected nitrogen-fixing bacteria were tested for gram-positive and gram-negative by using 3% KOH (Suslow et al., 1982). Bacterial cells from overnight grown culture were thoroughly mixed with a drop of KOH on a microscopic slide using a sterile toothpick. The appearance of a viscous and mucoid string upon raising the toothpick was the determining factor to differentiate bacteria as gram-negative. *Burkholderia glumae* and *Bacillus* sp. were used as controls for gram-negative and gram-positive bacteria, respectively.

Genomic DNA of bacterial isolates was extracted using the Sigma Aldrich’s GenElute Bacterial Genomic DNA Kit. The 16S ribosomal DNA sequence was amplified for partial identification of bacterial isolates using the following primers: fD1 (50-AGAGTTT-
GATCCTGGCTCAG-30) and rP2 (30-ACGGCTACCTTGT- TACGACTT-50) (Weisburg et al., 1991). For identification, BLAST (Basic Local Alignment Search Tool), from NCBI Gene Bank (www.ncbi.nlm.nih.gov), was used for homology search of 16S rDNA sequences in the DNA database.

3.2.6. Construction of GFP-tagged isolates

Two isolates, RRB I-6 and RRB I-18, were selected for labeling with green fluorescence protein (GFP) gene by triparental mating. GFP, a small-sized protein obtained from the jellyfish Aequorea victoria (Prasher et al., 1992), has now widely been used for colonization studies of PGPRs in rice and other crops (Hao and Chen, 2017; Liu et al., 2006; Zhu et al., 2002). In this study, donor bacteria strain Escherichia coli with GFP plasmid construct pBB2rpoDGFP1 (Barphagha and Ham, unpublished) (kanamycin-resistant (Km) at 50 μg L⁻¹), recipient bacteria (RRB I-6 and RRB I-18, nitrofurantoin-resistant (Nt') at 50 μg L⁻¹), and the helper strain E. coli HB101 (prK2013Tn7) were grown overnight in LB broth with suitable antibiotics at 30 °C. All bacteria were mixed in 1:1:1 ratio (volume/volume) with 500 μL each in a microcentrifuge tube, and 1.5 mL for each of the strains was taken as a control. The bacteria cultures grown in LB broth were centrifuged for 1 min. After discarding supernatant, the pellet was resuspended in 50 μL LB broth. The bacterial suspension was spotted on LB agar plate and incubated overnight at 30 °C. All the bacterial cells were harvested and resuspended in 1 mL LB broth and plated on LB agar plates supplemented with Km and Nt. The plates were incubated at 30 °C for 24 – 48 h to screen for successful transconjugants. Potential candidates were purified and further confirmed by observing green fluorescence under a fluorescence microscope.

3.2.7. Colonization and visualization of diazotrophic bacteria in rice plants

Rice seeds treated with GFP tagged strains (I-6-GFP and I-18-GFP) were allowed to grow for 2 weeks in falcon tubes with half strength Yoshida’s nutrient solution in a hydroponic
system. The presence or absence of I-6-GFP and I-18-GFP was determined by plating suspensions and counting the CFU on LB agar plates (supplemented with Km 50 μgL⁻¹, Nt 100 μgL⁻¹, Cyclohexamide (Cm) 40 μgL⁻¹). The presence of GFP-tagged bacteria on seeds was confirmed by vortexing treated seed in a microcentrifuge tube with 1 mL of sterile water followed by serial dilution plating on LB agar (with antibiotics). The plant samples were collected and lightly washed with sterile water. Roots and shoots were separated for each seedling. Rice roots were vortexed with 5 mL sterile water, serially diluted and plated on antibiotics containing LB agar to check the presence of bacteria on the root surface. The plant samples were surface-sterilized by 30 s treatment with ethanol (70% v:v) followed by 10 min treatment with 1.2 % sodium hypochlorite and rinsing with sterile water. The surface-sterilized shoots and roots were separately macerated in 5 mL sterile water by using sterile mortar and pestle and used for serial dilutions. 10 μL of each sample for each dilution were drop-placed on LB agar (antibiotics supplemented) with three replicated plates. The experiment was repeated and the average for six readings was recorded for the presence of bacteria in various parts of a rice seedling grown hydroponically.

The presence of GFP-tagged strains in roots and shoots was confirmed under a confocal laser scanning microscope (CLSM). Roots from 2-week-old seedlings (non-sterilized and surface-sterilized) and shoots (surface-sterilized) were stained with propidium iodide (10 μg/mL) for 10 min. The stained samples were placed on a microscopic glass slide with 0.6 % agarose solution (m:v), covered with a glass side and observed under CLSM to observe for bacterial fluorescence. GFP-tag-free plants were used as controls.

3.2.8. In vitro tests for other growth promoting traits of selected diazotrophs

Qualitative analysis for indole-3-acetic acid (IAA) production was performed using Salkoswki reagent (0.5 M FeCl₃, sterile distilled water and concentrated H₂SO₄ in a volume
proportion of 1:50:30) (Gordon and Weber, 1951). Overnight grown bacteria were inoculated into the nutrient broth with L-Tryptophan (1 mg mL$^{-1}$) and incubated at 30 °C for 7 days. The supernatant from bacterial culture was taken, and Salkowski reagent was added at 1:2 ratio. IAA production was determined by the development of light pink to red color. Bacterial strains were spot inoculated on Pikovskaya’s agar plates (with tri-calcium phosphate) and incubated at 30 °C for 3–7 days to evaluate phosphate-solubilization activity (PSB). The appearance of a clear zone around the bacterial spot was confirmation for P-solubilizing activity (Pikovskaya, 1948).

For siderophore production, Chrome Azurol S (CAS) blue agar plates (Schwyn and Neilands, 1987) with bromothymol blue as the indicator was used. The bacterial culture was spot inoculated on blue agar plates and incubated 2–6 days at 30 °C to observe clear orange halo zones around bacterial growth. All the isolates were also tested for antibacterial and antifungal activity against *Burkholderia glumae* and *Rhizoctonia solani* in a growth-inhibition assay as described by Shrestha *et al.* (2018). Freshly grown bacterial cultures with OD$_{600}$ = 0.1 (ca. 5×10$^8$ CFU/ml) were dropped on three spots on nutrient plates with the bacterial or fungal pathogen and incubated at 30 °C. The plates were examined for antibacterial activity (development of inhibition zone) after 48–72 h.

3.3. **Results**

3.3.1. **Isolation, screening and characterization of nitrogen-fixing bacteria**

More than 1,500 bacterial strains were isolated from the rice rhizosphere soil samples, of which, 186 bacterial isolates were initially selected based on morphology. The selected strains were further tested by *in vitro* screening for nitrogen-fixing activity, ammonia production, antifungal and antibacterial activities as well as siderophore production and phosphate solubilization activities (Table 3.1). In the nitrogen-free semi-solid medium, 18 isolates showed nitrogen-fixing activity (green to blue color change) after 3-10 days of incubation at 30 °C.
Based on the intensity of color change in N-fixing assay, 5 of the N-fixing positive bacteria were selected for further tests to evaluate their potential to promote rice growth. All five isolates, when reacted with Nessler’s reagent, changed the color of the nitrogen-free mixture from colorless to light yellow-brown, indicating a positive result for the ammonia production test (Figure 3.1B). Among the five isolates, RRB I-6 and RRB II-18 showed higher nitrogen-fixing and ammonia production activities (Figure 3.1). RRB I-6 and RRB II-18 also showed antagonistic activity against B. glumae, and none of the N-fixing isolates showed antifungal activity against R. solani (Table 3.1). The qualitative analysis of siderophore production revealed that all five nitrogen-fixing isolates were able to produce siderophores when incubated for 2–6 days at 30 °C, with RRB I-6 and RRB I-18 showing higher activity based on their larger orange halo zones (Table 3.1 and SFigure 3.1). In the assay for IAA production, RRB I-6 and RRB I-18 showed less IAA production ability, but RRB II-35, II-37 and III-4 showed strong IAA production activities (Table 3.1 and SFigure 3.2). All the isolates showed their potential to solubilize tricalcium phosphate in Pikovskaya agar plate with RRB II-35, II-37 and III-4 with highest phosphate solubilization activity (Table 3.1).

Table 3.1. Growth promoting features of isolated diazotrophic bacteria.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Nitrogen-fixing</th>
<th>Ammonia</th>
<th>Anti-bacterial</th>
<th>Anti-fungal</th>
<th>IAA</th>
<th>Siderophore</th>
<th>Phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRB I-6</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>RRB I-18</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>RRB II-35</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>+++</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>RRB II-37</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>RRB III-4</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

+, showing low activity; ++, showing moderate activity; ++++, showing strong activity; –, showing no activity
Figure 3.1. Isolates showing nitrogen-fixing (A) after 3 days and ammonia production (B) activity after 7 days. _Sinorhizobium meliloti_ was used as positive control and _Escherichia coli_ and without bacteria were used as negative controls.

3.3.2. Identification of bacterial isolates

All the five bacterial isolates having N-fixing activities were found to be gram-negative bacteria according to Ryu’s KOH test. The identification of all the potential diazotrophic isolates was based on the analysis of their 16S rDNA gene sequences. The bacterial sequences were compared with the NCBI GenBank database using BlastN tool, and matching species showing the highest similarity were recorded. Based on the gram staining and 16S rDNA sequence results, the bacterial isolates were characterized to be _Pseudomonas_ sp. and _Flavobacterium_ sp. (Table 3.2).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>3% KOH test</th>
<th>Species with highest homology match</th>
<th>% Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRB I-6</td>
<td>Gram-negative</td>
<td><em>Pseudomonas guariconensis</em></td>
<td>99.54</td>
</tr>
<tr>
<td>RRB I-18</td>
<td>Gram-negative</td>
<td><em>Pseudomonas guariconensis</em></td>
<td>99.54</td>
</tr>
<tr>
<td>RRB II-35</td>
<td>Gram-negative</td>
<td><em>Flavobacterium acidificum</em></td>
<td>96.5</td>
</tr>
<tr>
<td>RRB II-37</td>
<td>Gram-negative</td>
<td><em>Pseudomonas entomophila</em></td>
<td>96.25</td>
</tr>
<tr>
<td>RRB III-4</td>
<td>Gram-negative</td>
<td><em>Flavobacterium acidificum</em></td>
<td>97.6</td>
</tr>
</tbody>
</table>
3.3.3. Effect of bacterial strains on rice growth under the growth chamber conditions

In the growth chamber experiment, the results from replicate experiments were combined due to non-significant interaction between treatments and experiments. All the main and interaction effects of diazotrophs and nitrogen significantly affected plant growth at the $p < 0.05$ level. The effect of seed treatment with diazotrophs RRB I-6, RRB I-18, RRB II-35, RRB II-37 and RRB III-4 on plant height, SPAD score and dry weight of rice seedlings is shown in Table 3.3. Each of the bacterial isolates had different plant growth responses under N-free and N-present conditions. The results indicate that the presence of N had a significant effect on plant height and SPAD readings of 21-day-old seedlings. In the presence of N, all bacterial strains significantly stimulated the seedling growth in terms of plant height compared to both controls. Seed treatment with RRB II-35, RRB I-6 and RRB I-18, RRB III-4 and RRB II-37 significantly increased plant height by 13.4 %, 9.7 %, 9.4 %, 8.1 % and 6.1 %, respectively, compared to the without bacteria control (Table 3.3). The effect of inoculation was apparent in the absence of N; all diazotrophic isolates significantly increased plant height. The effect of the isolates on plant height was greater in the absence of N than when N fertilizer was added (Table 3.3). The plant height increase provided by three of five diazotrophic isolates in the absence of N was similar to the height of plants grown in the presence of N without bacteria (Table 3.3). Seed treatment with S. meliloti (positive control) significantly increased plant height and SPAD score compared to the control only in the absence of N (Table 3.3). Only RRB I-6 (under N present condition) and RRB I-6 and RRB II-35 (under N-free condition) showed significance increase in dry weight when compared to the without bacteria control (Table 3.3). Dry weight was higher for the control with N than for all the diazotrophic isolates under N-free condition (Table 3.3).

The comparison of SPAD scores gives an idea about leaf greenness and thus the relative chlorophyll and N content in the plants. The value for SPAD scores was significantly higher for
seedlings treated with RRB I-6 and RRB II-35 compared to the control in the presence of N and significantly higher for all isolates under the N-free condition (Table 3.3). SPAD scores were higher than the control under the N-free condition for all five isolates and the S. meliloti control (Table 3.3). The results from the growth chamber experiment indicate the efficacy of all isolates to increase plant height under the presence and absence of N nutrient. Only RRB I-6 increased dry weight in the presence of N and only RRB II-37 increased dry weight in the absence of N (Table 3.3).

3.3.4. Effect of bacterial strains on rice growth under greenhouse conditions

The greenhouse experiments were not combined due to significant interaction between bacteria and experiments. The growth promotion potential of the selected diazotrophic isolates in the greenhouse tests was variable for both N-free and N-present growing conditions (Table 3.4). When N was present, only RRB I-6 and RRB II-35 increased seedling height (10 % and 7.6 %) compared to the control. S. meliloti also increased seedling height. Under N-free condition, only isolate RRB II-37 significantly increased plant height (10.4 %) compared to the control (Table 3.4). Seed bacterization increased the SPAD score for all five isolates in the presence of N. However, only isolates RRB I-6 and RRB II-35 had a significant effect on SPAD scores compared to control under the N-free condition (Table 3.4). Four of five diazotrophs significantly enhanced dry weight under the N-present condition, whereas no isolate increased dry weight under the N-free condition.

In the second experiment, isolates RRB I-18, RRB I-6, RRB II-35 and RRB II-37 along with S. meliloti significantly enhanced plant height (increased by 15.1%, 15.9%, 13.5%, 8.0%, and 9.8% respectively) in comparison to the control under the N-present condition. In the absence of N, isolates RRB II-35 and RRB III-4 increased plant height compared with the control (Table 3.5). SPAD score was higher for seedlings treated with RRB I-6 and S. meliloti in
the presence of N (Table 3.5). Under the N-free condition, isolates RRB I-6, RRB III-4 and RRB I-18 along with S. meliloti significantly increased SPAD value compared with the control (Table 3.5). The results from the SPAD score indicated a higher value for I-6 under both conditions and higher activity of III-4 under the N-free condition (Table 3.5). Seed treatment with all the isolates significantly enhanced shoot dry weight compared to the control in the presence of N; however, no difference was observed for treatments under N-free condition (Table 3.5).

From both greenhouse trials, diazotrophic isolates RRB I-6 and II-35 were found to have a significant impact on growth parameters in the presence of N; however, their effects were not promising in the absence of N. Moreover, II-37 and III-4 showed variable effects in both greenhouse trials but seem to have the potential to promote rice growth under suitable growing environment. Plant height and weight were higher for the control plants in the presence of N than for plants inoculated with all five diazotrophic bacteria in the absence of N.

3.3.5. Construction of GFP-tagged isolates

The plasmid pBB2rpoDGFP1 was successfully transformed into Pseudomonas spp. RRB I-6 and RRB I-18. The GFP-tagged strains were highly similar to wild strains in terms of morphology and growth and still showed nitrogen-fixing activity on semi-solid N-free media, indicating no adverse effect of transformation on the isolates. The bacterial cells with plasmid were showing green fluorescence when the liquid culture on the microscopic glass slide was observed under a fluorescence microscope, confirming successful transformation of the GFP containing plasmid into the recipient strains (Figure 3.2). RRB I-18 showed visually brighter fluorescence and more transformed colonies compared to RRB I-6 (Figure 3.2).
Table 3.3. Influence of seed treatment with diazotrophs on the early growth stage of rice in the presence and absence of nitrogen under growth chamber conditions.

<table>
<thead>
<tr>
<th>Nitrogen</th>
<th>Bacteria</th>
<th>Plant height (cm)</th>
<th>% change in plant height</th>
<th>SPAD&lt;sup&gt;b&lt;/sup&gt; score</th>
<th>% change in SPAD&lt;sup&gt;b&lt;/sup&gt; score</th>
<th>Dry weight per plant (mg)</th>
<th>% change in dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>With N</td>
<td>Control</td>
<td>34.0 ± 1.731</td>
<td>c</td>
<td>20.0 ± 1.857</td>
<td>c</td>
<td>43.4 ± 6.294</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>I-18</td>
<td>37.2 ± 1.964</td>
<td>ab</td>
<td>20.8 ± 1.615</td>
<td>bc</td>
<td>45.7 ± 7.771</td>
<td>ab</td>
</tr>
<tr>
<td></td>
<td>I-6</td>
<td>37.3 ± 1.777</td>
<td>ab</td>
<td>21.7 ± 1.801</td>
<td>ab</td>
<td>48.6 ± 9.389</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>II-35</td>
<td>38.6 ± 2.328</td>
<td>a</td>
<td>22.7 ± 2.188</td>
<td>a</td>
<td>47.8 ± 8.782</td>
<td>ab</td>
</tr>
<tr>
<td></td>
<td>II-37</td>
<td>36.1 ± 3.292</td>
<td>b</td>
<td>20.7 ± 4.020</td>
<td>bc</td>
<td>45.6 ± 6.399</td>
<td>ab</td>
</tr>
<tr>
<td></td>
<td>III-4</td>
<td>36.8 ± 2.247</td>
<td>ab</td>
<td>20.3 ± 2.430</td>
<td>bc</td>
<td>45.7 ± 6.458</td>
<td>ab</td>
</tr>
<tr>
<td></td>
<td>&lt;i&gt;S. meliloti&lt;/i&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.8 ± 2.138</td>
<td>c</td>
<td>19.9 ± 1.873</td>
<td>c</td>
<td>49.5 ± 9.064</td>
<td>a</td>
</tr>
<tr>
<td>Without N</td>
<td>Control</td>
<td>28.3 ± 1.846</td>
<td>f</td>
<td>14.1 ± 1.551</td>
<td>e</td>
<td>28.1 ± 3.425</td>
<td>d</td>
</tr>
<tr>
<td></td>
<td>I-18</td>
<td>32.5 ± 2.709</td>
<td>cde</td>
<td>16.2 ± 2.840</td>
<td>d</td>
<td>29.5 ± 3.009</td>
<td>d</td>
</tr>
<tr>
<td></td>
<td>I-6</td>
<td>31.6 ± 2.780</td>
<td>de</td>
<td>16.5 ± 2.613</td>
<td>d</td>
<td>32.4 ± 4.144</td>
<td>cd</td>
</tr>
<tr>
<td></td>
<td>II-35</td>
<td>31.2 ± 2.467</td>
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<td>29.3 ± 3.534</td>
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<td>II-37</td>
<td>33.2 ± 3.590</td>
<td>cd</td>
<td>15.9 ± 2.348</td>
<td>d</td>
<td>36.6 ± 5.731</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td>III-4</td>
<td>33.0 ± 3.669</td>
<td>cde</td>
<td>15.7 ± 2.967</td>
<td>de</td>
<td>31.8 ± 4.703</td>
<td>d</td>
</tr>
<tr>
<td></td>
<td>&lt;i&gt;S. meliloti&lt;/i&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.3 ± 1.860</td>
<td>e</td>
<td>16.1 ± 1.768</td>
<td>d</td>
<td>28.8 ± 3.417</td>
<td>d</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (n=15) for each treatment. Mean values with different letters (Fisher’s LSD) are significantly different (P ≤ 0.05). The experiment was conducted in a 50 mL falcon tube under growth chamber in a controlled setup in the presence and absence of NH₄NO₃ in Yoshida’s nutrient solution. All the variables were measured 21 days after planting. Percent change for each variable is in comparison to its control. Rice plants treated with buffer solution (10mM MgCl₂) without any bacteria served as a control for both with N and without N condition.

<i>a</i>sinorhizobium meliloti

<i>b</i>Soil-Plant Analysis Development
Table 3.4. Influence of seed treatment with diazotrophs on the early growth stage of rice in the presence and absence of nitrogen under greenhouse conditions (experiment 1).

<table>
<thead>
<tr>
<th>Nitrogen</th>
<th>Bacteria</th>
<th>Plant height (cm)</th>
<th>% change in plant height</th>
<th>SPAD&lt;sup&gt;b&lt;/sup&gt; score</th>
<th>% change in SPAD score</th>
<th>Dry weight per plant (mg)</th>
<th>% change in dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>With N</td>
<td>Control</td>
<td>39.4 ± 3.642 cd</td>
<td>–</td>
<td>17.5 ± 2.248 ef</td>
<td>–</td>
<td>40.2 ± 3.751 e</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>I-18</td>
<td>39.1 ± 3.410 d</td>
<td>-1.0</td>
<td>19.1 ± 2.000 bcd</td>
<td>9.3</td>
<td>39.5 ± 4.463 e</td>
<td>-1.7</td>
</tr>
<tr>
<td></td>
<td>I-6</td>
<td>43.4 ± 3.094 a</td>
<td>10.0</td>
<td>22.8 ± 2.237 a</td>
<td>30.2</td>
<td>76.5 ± 5.379 a</td>
<td>90.1</td>
</tr>
<tr>
<td></td>
<td>II-35</td>
<td>42.4 ± 4.072 ab</td>
<td>7.6</td>
<td>19.4 ± 2.227 bc</td>
<td>11.0</td>
<td>68.3 ± 6.403 b</td>
<td>69.9</td>
</tr>
<tr>
<td></td>
<td>II-37</td>
<td>41.8 ± 2.653 abc</td>
<td>6.0</td>
<td>20.1 ± 1.828 b</td>
<td>14.9</td>
<td>45.9 ± 4.720 d</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td>III-4</td>
<td>40.0 ± 3.294 bcd</td>
<td>1.3</td>
<td>19.1 ± 2.828 bcd</td>
<td>8.7</td>
<td>46.4 ± 4.711 d</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>S. meliloti&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.8 ± 2.629 a</td>
<td>8.5</td>
<td>23.4 ± 2.285 a</td>
<td>33.9</td>
<td>55.6 ± 5.799 c</td>
<td>38.3</td>
</tr>
<tr>
<td>Without N</td>
<td>Control</td>
<td>25.3 ± 2.047 f</td>
<td>–</td>
<td>18.0 ± 1.472 def</td>
<td>–</td>
<td>22.7 ± 1.746 fg</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>I-18</td>
<td>25.2 ± 1.890 f</td>
<td>-0.3</td>
<td>18.6 ± 2.627 cde</td>
<td>3.4</td>
<td>20.8 ± 2.871 fg</td>
<td>-8.4</td>
</tr>
<tr>
<td></td>
<td>I-6</td>
<td>24.9 ± 1.661 f</td>
<td>-1.7</td>
<td>19.6 ± 1.958 bc</td>
<td>8.9</td>
<td>19.3 ± 1.764 g</td>
<td>-15.0</td>
</tr>
<tr>
<td></td>
<td>II-35</td>
<td>25.6 ± 2.555 ef</td>
<td>1.3</td>
<td>19.6 ± 1.281 bc</td>
<td>8.7</td>
<td>24.7 ± 1.272 f</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>II-37</td>
<td>27.9 ± 3.179 e</td>
<td>10.4</td>
<td>16.8 ± 1.518 f</td>
<td>-6.8</td>
<td>22.2 ± 0.713 fg</td>
<td>-1.9</td>
</tr>
<tr>
<td></td>
<td>III-4</td>
<td>24.6 ± 1.590 f</td>
<td>-2.8</td>
<td>18.6 ± 1.673 cde</td>
<td>3.4</td>
<td>19.3 ± 1.003 g</td>
<td>-14.7</td>
</tr>
<tr>
<td></td>
<td>S. meliloti&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.4 ± 1.863 f</td>
<td>-3.6</td>
<td>19.2 ± 2.015 bc</td>
<td>7.0</td>
<td>19.7 ± 2.202 g</td>
<td>-13.2</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (n=5) for each treatment. Mean values with different letters (Fisher’s LSD) are significantly different (P ≤ 0.05). The experiment was conducted for 30 days under greenhouse conditions in the presence and absence of NH₄NO₃ in Yoshida’s nutrient solution. Shoot dry weight was taken instead of the whole seedling to remove the variation caused by attached sand particles. Percent change for each variable is in comparison to its control. Rice plants treated with buffer solution (10mM MgCl₂) without any bacteria served as controls for both with N and without N condition.

<sup>a</sup> *Sinorhizobium meliloti*

<sup>b</sup> Soil-Plant Analysis Development
Table 3.5. Influence of seed treatment with diazotrophs on the early growth stage of rice in the presence and absence of nitrogen under greenhouse conditions (experiment 2).

<table>
<thead>
<tr>
<th>Nitrogen</th>
<th>Bacteria</th>
<th>Plant height (cm)</th>
<th>% change in plant height</th>
<th>SPAD$^b$ score</th>
<th>% change in SPAD score</th>
<th>Dry weight per plant (mg)</th>
<th>% change in dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>With N</td>
<td>Control</td>
<td>36.3 ± 2.620 d</td>
<td>–</td>
<td>16.1 ± 1.06 de</td>
<td>–</td>
<td>39.4 ± 3.186 c</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>I-18</td>
<td>41.8 ± 1.902 a</td>
<td>15.1</td>
<td>16.9 ± 0.956 bcd</td>
<td>4.5</td>
<td>47.1 ± 1.678 ab</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>I-6</td>
<td>42.1 ± 2.945 a</td>
<td>15.9</td>
<td>18.5 ± 0.881 a</td>
<td>14.6</td>
<td>49.5 ± 1.117 a</td>
<td>25.5</td>
</tr>
<tr>
<td></td>
<td>II-35</td>
<td>41.3 ± 2.259 ab</td>
<td>13.5</td>
<td>16.5 ± 0.786 bcde</td>
<td>2.1</td>
<td>47.2 ± 1.467 ab</td>
<td>19.6</td>
</tr>
<tr>
<td></td>
<td>II-37</td>
<td>39.2 ± 2.384 c</td>
<td>8.0</td>
<td>16.9 ± 0.857 bcd</td>
<td>5.0</td>
<td>47.6 ± 3.399 ab</td>
<td>20.6</td>
</tr>
<tr>
<td></td>
<td>III-4</td>
<td>37.7 ± 3.439 d</td>
<td>3.8</td>
<td>16.2 ± 0.622 cde</td>
<td>0.7</td>
<td>45.3 ± 2.791 b</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>S. meliloti$^a$</td>
<td>39.9 ± 2.371 bc</td>
<td>9.8</td>
<td>17.2 ± 1.127 b</td>
<td>6.7</td>
<td>47.8 ± 3.586 ab</td>
<td>21.1</td>
</tr>
<tr>
<td>Without N</td>
<td>Control</td>
<td>22.7 ± 1.980 g</td>
<td>–</td>
<td>16.0 ± 1.644 e</td>
<td>–</td>
<td>19.0 ± 1.709 d</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>I-18</td>
<td>24.1 ± 1.183 efg</td>
<td>6.1</td>
<td>16.9 ± 0.944 bcd</td>
<td>5.7</td>
<td>19.9 ± 0.876 d</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>I-6</td>
<td>22.8 ± 1.722 g</td>
<td>0.7</td>
<td>18.4 ± 1.142 a</td>
<td>15.0</td>
<td>19.8 ± 1.427 d</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>II-35</td>
<td>24.3 ± 1.504 ef</td>
<td>7.4</td>
<td>16.8 ± 1.317 bcde</td>
<td>4.7</td>
<td>20.4 ± 1.443 d</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>II-37</td>
<td>23.5 ± 1.300 efg</td>
<td>3.8</td>
<td>16.7 ± 1.298 bcde</td>
<td>4.5</td>
<td>19.1 ± 3.028 d</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>III-4</td>
<td>24.5 ± 1.439 e</td>
<td>8.0</td>
<td>17.0 ± 1.066 bcde</td>
<td>6.4</td>
<td>20.4 ± 1.120 d</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>S. meliloti$^a$</td>
<td>23.0 ± 1.246 fg</td>
<td>1.3</td>
<td>17.0 ± 1.06 bc</td>
<td>6.2</td>
<td>19.0 ± 1.112 d</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (n=5) for each treatment. Mean values with different letters (Fisher’s LSD) are significantly different (P ≤ 0.05). The experiment was conducted for 30 days under greenhouse conditions in the presence and absence of NH$_4$NO$_3$ in Yoshida’s nutrient solution. Shoot dry weight was taken instead of the whole seedling to remove the variation caused by attached sand particles. Percent change for each variable is in comparison to its control. Rice plants treated with buffer solution (10mM MgCl$_2$) without any bacteria served as controls for both with N and without N condition.

$^a$Sinorhizobium meliloti

$^b$Soil-Plant Analysis Development
3.3.6. Colonization of rice plants by GFP-tagged N-fixing bacteria

Results from Confocal Laser Scanning Microscope viewing revealed the successful GFP tagging of RRB I-6 and RRB I-18 isolates visible on the root surface of rice seedlings grown from inoculated seed (Figure 3.2). No green fluorescent bacteria were detected on the root surface of the control (non-inoculated) plants (Figure 3.2). The bright green dots (bacteria) on the surface of the rice roots indicate the cells of the diazotrophic isolates RRB I-6 and RRB I-18 that colonized the rice roots (Figure 3.2) although the image of the bacteria cells were not clearly distinguished due to green autofluorescence (excited by the blue light of microscope) from epidermis, chlorophyll, and other plants parts. However, the bacterial bright green dots were not detected inside the roots, stem and leaf parts of the inoculated rice plants when observed under CLSM (Figure 3.2).

The detection of GFP-tagged RRB I-6 and RRB I-18 isolates on the rice seeds, culture solution of the hydroponic culture system and plant parts was carried out by counting colony forming units (CFUs) on the LB agar plates (supplemented with Km, Nt, Cm) (Table 3.6). The rice seeds were initially treated with GFP-tagged strains with a concentration of ca. $2 \times 10^9$ CFU/ml. After 24 h, around $0.26 \times 10^7$ CFU/seed of RRB I-6 and $0.95 \times 10^7$ CFU/seed of RRB I-18 was found associated with the surface of rice seeds (Table 3.6). The GFP-tagged bacterial cells could be detected from the liquid culture used for the hydroponic growth of rice seedlings and from the surface of the rice roots. However, bacterial cells could not be isolated from the surface-sterilized root and shoot parts of the rice seedlings (Table 3.6). No bacterial cells were recovered from non-treated seeds and rice seedlings (root and shoot) on LB agar plates.

3.4. Discussion

In Louisiana, growers mainly depend on synthetic chemical fertilizers for adding N to the soil in rice fields. Application of synthetic N fertilizers adversely affects human health and the
environment. Nitrogen-fixing bacteria not only can supplement N to the rice plant through biological nitrogen fixation, but also enhance plant growth via regulating growth hormones, increasing nutrient uptake, and/or managing several abiotic and biotic stresses affecting plants (Glick, 2012; Kloeper, 1981). The search for potential diazotrophs with multiple growth-promoting mechanisms might help to reduce the application of synthetic N fertilizer without compromising growth and yield of crops. In this study, bacterial colonies were isolated from rhizosphere soil samples and further screened for nitrogen-fixing activities, of which five nitrogen-fixing isolates (from the genera Pseudomonas and Flavobacterium) were selected for growth chamber and greenhouse experiments to evaluate their potential to enhance rice growth.

Besides nitrogen-fixing assay, five selected isolates showed other plant growth-promoting traits. All of the selected bacterial strains showed the potential to produce a considerable amount of siderophores on CAS agar plates. The members of the genus Pseudomonas and Flavobacterium have been reported to compete with other bacteria and phytopathogens through competition for iron by siderophore production (Walitang et al., 2017). However, only RRB I-6 and RRB I-18 showed the antagonistic potential against B. glumae. All five isolates were positive for ammonia production, with RRB I-6 showing higher activity for ammonia production. Previous studies initially reported inability of Pseudomonas strains to fix nitrogen (Young, 1992). However, few strains from the genus Pseudomonas have been known

Table 3.6. Populations of GFP-tagged RRB I-6 and RRB I-18 detected from the treated seeds, culture solution, root surface and the inside of root and shoot tissues of 14 day-old rice seedlings.

<table>
<thead>
<tr>
<th>Source of isolation</th>
<th>Control</th>
<th>RRB I-6</th>
<th>RRB I-18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed</td>
<td>–</td>
<td>0.26 * 10^7 CFU/seed</td>
<td>0.95 * 10^7 CFU/seed</td>
</tr>
<tr>
<td>Culture solution</td>
<td>–</td>
<td>0.17 * 10^4 CFU/ml</td>
<td>2.9 * 10^4 CFU/ml</td>
</tr>
<tr>
<td>Root (surface)</td>
<td>–</td>
<td>0.19 * 10^6 CFU/root</td>
<td>0.43 * 10^6 CFU/root</td>
</tr>
<tr>
<td>Root (inside)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Shoot (inside)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
Figure 3.2. Visualization of GFP-tagged bacteria in liquid culture (left) and rice plant (right). Diazotrophic bacteria *Pseudomonas* sp. RRB I-6 and RRB I-18 tagged with *GFP* were observed under a fluorescence microscope (left column). Bacterial cells in liquid culture on a glass slide were looking like green dots; however, no fluorescence was observed on control (a liquid culture with RRB I-6 *GFP*-free) were observed on root surface using confocal microscopy. The presence of *GFP*-tagged RRB I-6 and I-18 was confirmed under a confocal laser scanning microscope (CLSM) (middle and right columns). Plant samples were stained with propidium iodide to stain the cell walls. Bright green dots were observed on the surface of rice roots, indicating the presence of *GFP*-tagged bacteria. Plants without bacterial inoculation were served as the negative control.
for their nitrogen-fixing ability, including P. stutzeri and P. azotifigens (Hatayama et al., 2005; Ma et al., 2016). Our study showed the potential of another Pseudomonas sp. RRB I-6 and I-18 to fix nitrogen and to produce ammonia. In vitro tests for growth promoting traits confirmed the potential of the selected nitrogen-fixing bacteria to promote plant growth through multiple plant growth promoting mechanisms.

In the present study, multiple trials were conducted under growth chamber and greenhouse conditions to test the efficacy of diazotrophs isolated from rice field soil as a seed treatment on enhancing initial seedling growth of rice. In the growth chamber experiment, rice seeds treated with all five diazotrophs exhibited increased in plant height compared to the control, both in the presence and absence of N, but only one increased dry weight. Plants treated with diazotrophs had higher SPAD values along with increased plant height compared to the control for both conditions. The diazotrophs had larger increases for SPAD in the absence of N. A previous study reported similar SPAD readings with non-significant differences between treatments for 4-week rice plants; however, treatment with Citrobacter sp. showed the difference in SPAD readings along with enhanced plant growth and root nitrogen content after 56 days (Hongrittipun et al., 2014). Previous investigations have reported reduced nitrogen-fixing activity due to chemical fertilization (ammonium sulfate) for Azotobacter in maize (Dobereiner, 1974) and also in Gluconacetobacter in sugarcane (Muthukumarasamy et al., 1999). Moreover, synthetic chemicals can limit the diversity of diazotrophs at the genetic level (Caballero-Mellado and Martinez-Romero, 1994). Despite the lower rate increases in SPAD for the plants grown in the presence of synthetic N, treatments with diazotrophs increased plant height and chlorophyll content compared to the control in the presence of N. The growth promotion activities by diazotrophs in the presence of N indicate potential additive effects of the diazotrophic isolates...
and the added nitrogen in enhancing seedling height. The increases in plant height and SPAD scores could be the result of extra N from biological fixation, increased uptake of N or other nutrients, production of phytohormones or other growth promoting mechanisms. All the tested isolates also showed the ability to produce ammonia, an additional growth promoting trait.

Several strains of PGPRs including *Pseudomonas* sp. have potential to produce 1-Aminocyclopropane-1-carboxylate (ACC) deaminase which can convert the ethylene precursor ACC to ammonia and reduce the stress caused by plant-produced ethylene (Arshad *et al.*, 2007; Shaharoona *et al.*, 2007). Also, *in vitro* tests showed the potential of the isolates to produce siderophores and indole acetic acid (IAA).

The *Pseudomonas* strain RRB I-6 was the most promising in terms of promoting plant growth under both growth chamber and greenhouse conditions. Diazotrophic isolates *Pseudomonas* strain RRB II-37 and *Flavobacterium* strains RRB II-35 and RRB III-4 were also able to enhance seedling growth in the growth chamber and greenhouse trials, especially under the N-free condition. A previous investigation reported increases in seed germination, shoot length and dry weight, when rice seeds were treated with *Flavobacterium* sp. IC31–28 (Walitang *et al.*, 2017). Also, seed treatment with *P. guariconensis* was reported to control collar rot disease and enhance germination and seedling growth of peanut through phytohormone production and enhancement of the uptake of N, P, K and Zn (Patel *et al.*, 2015).

When rice seedlings treated with *GFP*-tagged RRB I-6 and RRB I-18 were observed under CLSM, bacterial cells with bright green fluorescence were detected only on the root surface but not the inside rice tissues. The microscopy results indicate the ability of bacteria to colonize the root surface in the rhizosphere of the rice plants. The presence of bacteria detected by microscopy was consistent with the CFU counting experiment. *GFP*-tagged bacterial colonies
of *Pseudomonas* strains I-6 and I-18 were observed on the LB agar plates (added with Km, Nt, and Cm) only from the culture medium and the root surface. No bacterial colonies were observed for control plates representing non-inoculated rice plants. A high number of bacterial colonies were observed on plates from the rice seeds treated with *GFP*-tagged isolates. CFU counting from treated rice seeds after 24 h indicates the success of seed treatment. Also, bacteria were found to survive on the treated rice seeds even when tested after several days of storing at 4 °C (data not shown). This result indicates the ability of RRB I-6 and RRB I-18 to survive when used as a seed treatment, which is a very efficient method to deliver potent bacterial strains to the field.

The count for CFU/ml in the culture solution was $0.17 \times 10^4$ CFU/ml and $2.9 \times 10^4$ CFU/ml for RRB I-6 and RRB I-18, respectively and seemed to be slightly low compared to the initial bacterial cells attached on the rice seeds. The bacteria multiplication might have been affected by limited surface area in the 50 mL tubes. The growth and increased activity of bacteria in a limited space may have influenced the pH and other conditions in the culture solution of the hydroponic system used, and thus limited the bacterial multiplication. The bacterial strains tagged with GFP have been utilized in many studies to study the colonization potential of *Bacillus* sp., *Rhizobium* sp., and *Burkholderia* sp. in rice plants (Liu *et al.*, 2006; Singh *et al.*, 2009). Our study also highlights the importance of GFP technique to observe the colonization potential of beneficial bacteria. Diazotrophs RRB I-6 and I-18 should be further studied starting from the time of seed treatment to rice plant maturity under various environments to evaluate the ability of bacteria to multiply, colonize and survive under different conditions. Imaging was difficult for the interior parts of rice root, stem and leaf under CLSM. The clearing agent to
prepare microscopic slides might help to obtain clear images inside plant parts for the detection of the presence/absence of bacterial cells.

*Pseudomonas* sp. RRB I-6 may have an excellent potential to enhance rice growth at the early vegetative growth stage. The amount of fixed N by these diazotrophs may be low; however, treatment of plants with these isolates might assist in initial seedling establishment via root and shoot elongation, efficient utilization of nutrients and increased tolerance to adverse biotic and abiotic stress. Further studies should include the evaluation of these beneficial bacteria in variable rates and in combination with nitrogen fertilizers under different field conditions. Furthermore, the application of multiple bacteria could give better results in terms of plant growth and yield due to additive effects. Inoculation with bacterial combinations (compatible isolates) was reported to promote rice growth through various mechanisms (Bashan *et al*., 2004). The amount of N fixed by diazotrophic bacteria cannot replace chemical fertilizers; however, utilization of the potential diazotrophs with several growth-promoting traits might aid in sustainable agriculture.
CHAPTER IV. CONCLUSIONS

In this study, rice-associated bacteria (RAB) with potential biocontrol activities were evaluated for their efficacy in managing two important rice diseases of Louisiana, bacterial panicle blight (BPB) and sheath blight (ShB). The antagonistic potential of RABs were evaluated in vitro against the bacterial pathogen Burkholderia glumae and the fungal rice pathogen, Rhizoctonia solani and further evaluated under field and greenhouse conditions. For management of BPB, the efficacy of selected antagonistic Pseudomonas strains in managing BPB could not be determined due to the low disease pressure of BPB in two field trials. However, lack of effective management options, negative impacts of chemical pesticides and diversity of the pathogen makes BPB a potential threat to rice cultivation. Therefore, the studies on developing better and more viable biocontrol agents should be continued in Louisiana rice production.

For the management of ShB, Bacillus strain REB711 exhibited antagonistic activity against the ShB pathogen R. solani in a growth inhibition assay and further reduced the disease symptoms in the field and greenhouse studies. REB711 could be a potential biological control agent to suppress ShB and delay its epidemics. As REB711 can reduce disease by foliar or seed treatment, future studies should focus on the combined effect of seed treatment and foliar application of REB711 under the field conditions and also on the development of stable and durable formulation. Effective formulation of biocontrol agents is crucial in delivering to the field via foliar or seed treatment. Moreover, REB711 could be applied in combination (if compatible) with low rates of existing fungicides and might also be useful when combined with other methods through an integrated approach. Furthermore, the characterization of the antimicrobial compounds from REB711 and the study of genes responsible for antifungal activity in REB711 could help developing new chemical compounds.
The study on growth promotion of rice seedlings focused on isolation and screening of rice rhizobacteria for their nitrogen-fixing ability. The seed treatment with *Pseudomonas* sp. RRB I-6 showed potential to promote the growth of rice seedling under growth chamber and greenhouse conditions. All the five diazotrophs were positive for ammonia production, siderophore production, IAA production, and phosphate solubilization. *Pseudomonas* sp. RRB I-6 and RRB I-18 also showed antagonistic activity against *Burkholderia glumae* (pathogen of bacterial panicle blight). Increase in plant height and Soil-Plant Analysis Development (SPAD) scores could be a result of increased fixation of N or other nutrients or production of phytohormones. The efficacy of these candidate isolates was tested only in the presence and absence of NH$_4$NO$_3$. Evaluation of these candidates with variable forms and variable rates of nitrogen fertilizer may provide the best combination of diazotroph and nitrogen fertilizer for the field application.

This research highlights that biological agents (biocontrol and growth-promoting agents) may offer eco-friendly alternatives to the use of agrochemicals to manage the disease and promote the growth of rice plants. Further studies should focus not only on the screening of effective strains but also to improving their efficacy and durability under various field conditions. This is possible through the study of their biology, mechanisms, interaction with the plant and microbial ecology in soil and rhizosphere. Our findings will be useful in developing commercial products that can be directly used by farmers for promoting rice growth (leading to minimizing the use of commercial fertilizers) and suppressing major rice diseases (leading to minimizing the use of chemical pesticides).
LITERATURE CITED


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disinfectants. *Phytopathology* 90.


68


Schippers, B., Bakker, A.W. and Bakker, P.A.H.M. (1987) Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices,


## APPENDIX A. SUPPLEMENTARY FIGURES AND TABLES

### Supplementary Table 2.1. List of antagonistic bacteria (Ham’s Lab)

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Bacterial isolates</th>
<th>Inhibition area (mm²) <em>(B. glumae)</em></th>
<th>Inhibition area (mm²) <em>(R. solani)</em></th>
<th>Species identified</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RPB.NT 1</td>
<td>206.57 ± 7.08</td>
<td>not effective</td>
<td>Pseudomonas parafulva</td>
<td>Tony and Nan</td>
</tr>
<tr>
<td>2</td>
<td>RPB.NT 2</td>
<td>145.67 ± 14.02</td>
<td>not effective</td>
<td>Pseudomonas parafulva</td>
<td>Tony and Nan</td>
</tr>
<tr>
<td>3</td>
<td>RPB.NT 3</td>
<td>164.77 ± 18.64</td>
<td>not effective</td>
<td>Pseudomonas parafulva</td>
<td>Tony and Nan</td>
</tr>
<tr>
<td>4</td>
<td>RPB.NT 4</td>
<td>209.21 ± 11.43</td>
<td>not effective</td>
<td>Pseudomonas parafulva</td>
<td>Tony and Nan</td>
</tr>
<tr>
<td>5</td>
<td>RPB.NT 5</td>
<td>207.51 ± 14.50</td>
<td>not effective</td>
<td>Pseudomonas parafulva</td>
<td>Tony and Nan</td>
</tr>
<tr>
<td>6</td>
<td>RPB.NT 7</td>
<td>190.69 ± 14.50</td>
<td>not effective</td>
<td>Pseudomonas parafulva</td>
<td>Tony and Nan</td>
</tr>
<tr>
<td>7</td>
<td>RPB.NT 8</td>
<td>180.01 ± 20.80</td>
<td>not effective</td>
<td>Pseudomonas parafulva</td>
<td>Tony and Nan</td>
</tr>
<tr>
<td>8</td>
<td>RPB.NT 9</td>
<td>175.86 ± 22.63</td>
<td>not effective</td>
<td>Pseudomonas parafulva</td>
<td>Tony and Nan</td>
</tr>
<tr>
<td>9</td>
<td>RPB.NT 16</td>
<td>71.26 ± 8.98</td>
<td>not effective</td>
<td>Pseudomonas parafulva</td>
<td>Tony and Nan</td>
</tr>
<tr>
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<td>RPB.NT 17</td>
<td>85.95 ± 6.60</td>
<td>not effective</td>
<td>Pseudomonas parafulva</td>
<td>Tony and Nan</td>
</tr>
<tr>
<td>11</td>
<td>RPB.NT 21</td>
<td>75.55 ± 6.26</td>
<td>not effective</td>
<td>Pseudomonas parafulva</td>
<td>Tony and Nan</td>
</tr>
<tr>
<td>12</td>
<td>RPB.NT 26</td>
<td>82.93 ± 5.01</td>
<td>not effective</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>13</td>
<td>REB 711</td>
<td>66.76 ± 17.65</td>
<td>53.93 ± 7.71</td>
<td>Bacillus amyloliquefaciens</td>
<td>Surendra Osti</td>
</tr>
<tr>
<td>14</td>
<td>REB 712</td>
<td>76.71 ± 11.44</td>
<td>not effective</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>15</td>
<td>REB 713</td>
<td>not effective</td>
<td>not effective</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>16</td>
<td>REB 714</td>
<td>69.51 ± 10.20</td>
<td>68.59 ± 10.43</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>17</td>
<td>REB 715</td>
<td>not effective</td>
<td>32.46 ± 4.80</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>18</td>
<td>REB 716</td>
<td>not effective</td>
<td>not effective</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>19</td>
<td>REB 717</td>
<td>not effective</td>
<td>not effective</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>20</td>
<td>REB 718</td>
<td>not effective</td>
<td>19.90 ± 1.81</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>21</td>
<td>REB 719</td>
<td>not effective</td>
<td>39.53 ± 7.71</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>22</td>
<td>RRB 982</td>
<td>104.98 ± 12.81</td>
<td>69.38 ± 11.34</td>
<td>Bacillus subtilis</td>
<td>Katherine</td>
</tr>
<tr>
<td>23</td>
<td>RRB 983</td>
<td>93.86 ± 12.17</td>
<td>44.51 ± 8.62</td>
<td>Bacillus amyloliquefaciens</td>
<td>Katherine</td>
</tr>
<tr>
<td>24</td>
<td>RRB 984</td>
<td>87.44 ± 18.86</td>
<td>60.74 ± 15.03</td>
<td>Bacillus amyloliquefaciens</td>
<td>Katherine</td>
</tr>
<tr>
<td>25</td>
<td>RRB 985</td>
<td>not effective</td>
<td>69.64 ± 20.42</td>
<td>Bacillus subtilis</td>
<td>Katherine</td>
</tr>
<tr>
<td>26</td>
<td>RRB 1043</td>
<td>56.81 ± 26.29</td>
<td>not effective</td>
<td>Bacillus subtilis</td>
<td>Katherine</td>
</tr>
<tr>
<td>27</td>
<td>RRB 1044</td>
<td>233.13 ± 17.05</td>
<td>not effective</td>
<td>P. plecoglossicida</td>
<td>Katherine</td>
</tr>
<tr>
<td>28</td>
<td>RRB 1046</td>
<td>188.89 ± 42.75</td>
<td>42.41 ± 15.45</td>
<td>Pseudomonas putida</td>
<td>Katherine</td>
</tr>
<tr>
<td>29</td>
<td>RRB 1047</td>
<td>286.54 ± 34.25</td>
<td>not effective</td>
<td>Pseudomonas putida</td>
<td>Katherine</td>
</tr>
<tr>
<td>30</td>
<td>RIB3-3</td>
<td>not effective</td>
<td>not effective</td>
<td>Bacillus sp.</td>
<td>Katherine</td>
</tr>
<tr>
<td>31</td>
<td>RIB2-14</td>
<td>not effective</td>
<td>19.90 ± 1.81</td>
<td>Burkholderia glumae</td>
<td>Katherine</td>
</tr>
<tr>
<td>32</td>
<td>RIB4-22</td>
<td>73.30 ± 10.76</td>
<td>39.53 ± 7.71</td>
<td>Bacillus sp.</td>
<td>Katherine</td>
</tr>
<tr>
<td>33</td>
<td>RIB-6</td>
<td>not effective</td>
<td>13.09 ± 4.99</td>
<td>Burkholderia sp.</td>
<td>Katherine</td>
</tr>
<tr>
<td>34</td>
<td>RIB1-20</td>
<td>not effective</td>
<td>23.82 ± 6.10</td>
<td>Burkholderia glumae</td>
<td>Katherine</td>
</tr>
<tr>
<td>35</td>
<td>RAB 14R</td>
<td>N/A</td>
<td>65.44 ± 11.12</td>
<td>Bacillus amyloliquefaciens</td>
<td>Bishnu Karki</td>
</tr>
</tbody>
</table>

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Supplementary Table 2.2. Evaluation of Bacillus strains to reduce ShB infestation respectively under the field condition in 2017/18

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Disease score ± SD</td>
<td>Average disease reduction %</td>
</tr>
<tr>
<td>Non-inoculated</td>
<td>3.8 ± 0.419 d</td>
<td>48.8</td>
</tr>
<tr>
<td>RRB985/WC</td>
<td>4.9 ± 1.578 bcd</td>
<td>33.6</td>
</tr>
<tr>
<td>RRB985</td>
<td>5.5 ± 1.170 bc</td>
<td>25.8</td>
</tr>
<tr>
<td>REB711/WC</td>
<td>5.2 ± 1.191 bcd</td>
<td>29.2</td>
</tr>
<tr>
<td>REB711</td>
<td>5.2 ± 0.904 bcd</td>
<td>30.2</td>
</tr>
<tr>
<td>RAB14R/WC</td>
<td>5.5 ± 0.443 bc</td>
<td>25.8</td>
</tr>
<tr>
<td>RAB14R</td>
<td>6.1 ± 1.189 ab</td>
<td>18.0</td>
</tr>
<tr>
<td>Quadris</td>
<td>3.9 ± 1.086 cd</td>
<td>47.1</td>
</tr>
<tr>
<td>Non-treated</td>
<td>7.4 ± 0.533 a</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Two experiments (trials) were conducted. Means of disease score for each treatment followed by different letters are significantly different at p<0.05.

Supplementary Figure 2.1. Effect of form of culture media (solid agar plate vs. liquid broth) used for bacterial growth in suppression of ShB. Antagonistic RABs initially grown on solid LB agar plate and liquid broth were suspended in 10mM MgCl\(_2\) buffer solution to prepare bacterial suspension, which was later sprayed on the rice panicles to evaluate ShB suppression.
**Supplementary Table 2.3.** Evaluation of *Bacillus* strains to reduce disease severity of ShB under the greenhouse condition.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Trial 1</th>
<th></th>
<th></th>
<th>Trial 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Disease score ± SD</td>
<td>Average disease</td>
<td>Disease score ± SD</td>
<td>Average disease</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>reduction %</td>
<td></td>
<td>reduction %</td>
<td></td>
</tr>
<tr>
<td>Non-inoculated</td>
<td>0</td>
<td>d</td>
<td>100.0</td>
<td>0</td>
<td>d</td>
</tr>
<tr>
<td>RRB 985</td>
<td>4.12 ± 0.522</td>
<td>a</td>
<td>3.1</td>
<td>2.66 ± 0.932</td>
<td>bc</td>
</tr>
<tr>
<td>REB711</td>
<td>2.08 ± 0.729</td>
<td>c</td>
<td>51.6</td>
<td>2.2 ± 0.374</td>
<td>c</td>
</tr>
<tr>
<td>RAB14R</td>
<td>3.34 ± 0.688</td>
<td>b</td>
<td>21.9</td>
<td>3.22 ± 0.217</td>
<td>ab</td>
</tr>
<tr>
<td>Non-treated</td>
<td>4.26 ± 0.483</td>
<td>a</td>
<td>0</td>
<td>3.94 ± 0.873</td>
<td>ab</td>
</tr>
</tbody>
</table>

Two experiments (trials) were conducted. Means of disease score for each treatment followed by different letters are significantly different at p<0.05.

**Supplementary Table 2.4.** Evaluation of *Bacillus* strains to inhibit lesion development on leaf sheath and blades under the greenhouse condition.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Trial 1</th>
<th></th>
<th></th>
<th>Trial 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lesion length ± SD</td>
<td>Average lesion</td>
<td>Lesion length ± SD</td>
<td>Average lesion</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>reduction %</td>
<td></td>
<td>reduction %</td>
<td></td>
</tr>
<tr>
<td>Non-inoculated</td>
<td>0</td>
<td>c</td>
<td>100</td>
<td>0</td>
<td>d</td>
</tr>
<tr>
<td>RRB 985</td>
<td>3.38 ± 0.497</td>
<td>a</td>
<td>15.5</td>
<td>2.14 ± 0.684</td>
<td>b</td>
</tr>
<tr>
<td>REB711</td>
<td>1.48 ± 0.597</td>
<td>b</td>
<td>63</td>
<td>1.52 ± 0.277</td>
<td>c</td>
</tr>
<tr>
<td>RAB14R</td>
<td>3.24 ± 0.991</td>
<td>a</td>
<td>19</td>
<td>2.7 ± 0.274</td>
<td>a</td>
</tr>
<tr>
<td>Non-treated</td>
<td>4 ± 0.667</td>
<td>a</td>
<td>0</td>
<td>3 ± 0.490</td>
<td>a</td>
</tr>
</tbody>
</table>

Two experiments (trials) were conducted. Means of lesion length for each treatment followed by different letters are significantly different at p<0.05.
Supplementary Figure 2.2. Disease symptoms of ShB on rice plants under the greenhouse condition. Rice plants treated with REB711 (A) and Non-treated control (10 mM MgCl₂) (B)
Supplementary Figure 3.1. Siderophore production activity by selected potential diazotrophs

Supplementary Figure 3.2. Siderophore production activity by selected potential diazotrophs
Supplementary Figure 3.3. Growth promotion by diazotrophs under greenhouse condition (trial 1) under N-present condition.

Supplementary Figure 3.4. Growth promotion by diazotrophs under greenhouse condition (trial 2) under N-present condition.
APPENDIX B. 16S RDNA SEQUENCES OF NITROGEN-FIXING BACTERIA

RRB I-6
Pseudomonas guariconensis strain PCAVU11 16S ribosomal RNA, partial sequence
Sequence ID: NR_135703.1
Identity: 99.54%
Coverage: 100%

CTACGGGCTACCTTGTACGACTTCCACCCCAGTGATCATCACACCGCAGCGTTGGAACCGTC
CTCCCAGTTAGAAGCATGCTACTTTCTGGTGCAACCACCTCCCATGATGTGACCTGCC
GGTGTACAGGGCCGAGCGTATCCACCACGACATTCTGATTACCGCATTAAGCT
CGATTTCCGACTTCCGAGCTGGAACTGAGATCTGCCAGACCTCAGTCGAGTTGCAGT
GTGATTAGCTCTCACGCTTTAGGTCCCCACCAGTGACGTGCTTGAATTAAAGGAC
AAGGTTTGCCTGGTGTTACGGGACTTAAACCCACATCTACGACACGAGCTGACGAC
AGCCATGCAAGGCTGGTCAAGGACCACACTCCATCCTGGAACGGGACCCAAATCCATCT
CTCTGCAATGCTAAAGGCCTGTATAGTTCTTCGCTTGGTTCGATATTAAACACATGCTC
CCACAGGCTTACCAAATTCGTTAGCTGCCACTAATCCTAAGGGATTCACAAC
GGCTAGTGGACCATCGTTTACGGCGTGCTACAAGGGATACGGGCTAATCCTGTTT
CTGGCGCCTCAGGCTCGTATACGCTTACGCCAGGTGTCGCTTGCCCTGCCACTGTG
GGTTCTCTGCTATATCTACGATTTACCCGCTACTACAGGAAAATCCCACACCCACCT
ATACCTAGCTGCCAGTTTTGAGATGCTATCCAGCGGACCCCGCCTTACACA
TCCAATTAACGGAACCACCTACGCGGCTTACGCGCAGTTACAGCGGCTAATTC
GCCAACCTCTGTTATACCGCGGCTGCTTGGCGACAGTAGTTAGGCCGTGTTATCTGGTCG
GTAACGTCAAACAGCAAGGTATATTAACTACTACTGCCNTTCCTCACCACATTAAGGTGCT
TTACAAATCC

RRB I-18
Pseudomonas guariconensis strain PCAVU11 16S ribosomal RNA, partial sequence
99.54 % identity and 100% coverage
Sequence ID: NR_135703.1
Identity: 99.54%
Coverage: 100%

ATGACGGGCGANCTTGCTCTTGTGNTTCCNCGCAGGCGGACGCGGTGAGTAATGCTANGAATC
TGCCCTGGAAAGGGGACACGCTATCCGAAAGGAGCGCTAATACCGGATACGTCCTAGCT
CGGGAGAAGGGNGGAGCTCTTCGACCTACGCTATCNCTAGANCTTAGGTGCGNTAC
TAGCTANTTTGGNAGGNAANGGCTCACACNNCGGACAGATCGNNACACTGTTGCTGNA
GGATGATCANCNTGCACCTGGAACCTGAGACACGGTGTCGAGACTCTAGCCAGGCCAGCAG
ATCGGGGAAATTTGGCAATGGGCCNAAGCTGGAATCCAGACCCGCTACTTGCGTGCGTGA
AGAAGGTCCTTCTGATTGTAAGGCTGAGGAGAGGGCAGTAGATTAA
ACCTTGGCTTGTGTTAGGCTTGCAACGAGAATAAGGGACAGGGCCAGCAGCAGCAG
CCGCGGTAATACAGAGGGTGCAACGGTAAATCGGAATTACTGGGCGTAAAGCGCGC
GTAGGGTGCTTCCGTAAAGGGATGTAAGAACCCCGCGCTCAACCTGGGAAGTCG
TCCAAAACGGGCGAGCTAGAGTATGATGGAGGGTTGCTGGAATTCTCTGTGTAGCG
TGAAATGCGTACATATAAGGAAGCAACCCAGTGGCCGAAAGCCGACCACCTGGACTGA
TACTGACATCTGAGTCGGAAGCCTGGGGAGCAAACAGGATTAGATACCCTGGNAG
NCCACGCGTAACAGGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGCGCA
GCTAACGCATTAAGTTGAGCCGCTTGGAAG

**RRB III-4**

*Flavobacterium acidificum* strain LMG 8364 16S ribosomal RNA, partial sequence
Sequence ID: NR_104962.1
Identity: 97.6%
Coverage: 100% (high score)

**Pantoea agglomerans** strain DSM 3493 16S ribosomal RNA, partial sequence
Sequence ID: NR_041978.1
Identity: 97.6%
Coverage: 100%

TACCTTGNTACGACTTCACCCANTCATGAATCACAAAGTGGTANGCNCCCTCCCNA
AGGNTANGCTACCTACTTCCTTTTGCAACCCACTGCCNTGTTGCTGAGCGCGGTGTGT
ACAAAGGCNNGGAAACGATATCCACTACGCGACATTCTAGTACGAGGATTACCC
GACTTCACGAGTCGACCCGATCCCGAGTCGACGACGACGACCTTTATGAGGT
CCGCTTTGCTCTCAGGAGGCTCGTTTCTCTCTTTGTATGCGCCATTGTAGACGACG
CCTACTGGTAAGGGCCATGATGACCTGAGCTGTCATCCCCACCTTCTCTCGGTTTTATC
ACGCGACTCTCTTTGAGTTCCGCTTGCCAAAACTGCTCCCGGTTTTAC
CTCGTTGCGGAGCTAAAACCAACACATTCAACAACAGGCTGACGACGACGATGCAG
CACCCTGCTCNGNTTCCGAGGCATACGACTCTGTCGCTGCCNNAATTCCCTGGATG
TAAGAGTAGTAAGGTCTTCTCGGCTGGCATCGAATTAAACACATGCTCCAGGCTTT
GTGCCGCGCCCGTGCAATTCTATTGATGTATTAACCTTGGCGCCGCTTCCCAACGCG
GTCGACTTAAACCGGTAGCTCCCGGAAGCCACTTCAAGGAACCAACCTCAAAGTC
GACAATCGTAAACGCGTGAGCTAACCAGGATATCTAATCTGGTGGCTCACCACGTT
CCGACATGACTTCGTTCTCTTTCGAGGAGGCGCGCTTCGACCAGGTATCTCC
GATCTCTACGCTATTCCAGGCTACACTGGAATTCTACCCCTCCTACGAGACTCAA
GCCTGCGACTTCAAACTAAGTCCAGTATGTCAGGTCAGGTCAGGTCAGGTCAGG
AACAGACGCCTGCGCTTTACGCGCCATTAATCCGGACTGACACCCTC
CGTCAATTAGCGGGCTGCGCCAGGAGTTAGCCGCTTTCTCTGGCGTGAG
CAATCNATGCGGTTAATACCCGNCCTACCTCCCGCGTAAGAGTACTTTACAA
C

**RRB II-37**

*Pseudomonas entomophila* L48 16S ribosomal RNA, partial sequence
Sequence ID: NR_102854.1

*Pseudomonas guariconensis* strain PCAVU11 16S ribosomal RNA, partial sequence
Sequence ID: NR_135703.1

*Pseudomonas mosselii* strain CFML 90-83 16S ribosomal RNA, partial sequence
Sequence ID: NR_024924.1  
Identity: 96.25%  
Coverage: 100%

ATTACTAGCGATTCCGACTTCACGCACNCTCGAGTTGNAGACTGCGATCCGGACTANNA
NCNGNTTGTGAGATTAGNTCCACCTCGCGTTNTGGCAACCCTCTGGGACCGACCACATT
GGANCACGAGGCTAGCCAGCGGAAGGGGCATGACTTGACNTCTACCTCCCCAC
CTTCTCGGNTTGTKCACCAGGCACTTCTCCTTTANAGNGCCCAACCAGCTGACTGGNA
ACTAACAGCAAGGGNTGCGACTTACCGGACTTAAACCACATCTACGACAGCA
NTNGACGCACAGCCATGCGACACTGTGTCAAGTCCGAAAGCCACAAATCTCATT
CTGGAAAGTTCTCTGATGTCAAGGCCCCTGGAANGGTCTTCTCCTGCGTCGTCCGAATTA
AACCACATGCTCCACCGCTTGTGCGGCCCCCCTCAATTTGAGTTTAACTTGGC
CGGCGTCGACTCCCAGGGCCTCAGCTCTTAGCTGGCAGCCACTAAAATCTCAA
GAATCCTCAAGGCTAGTGGACATCTTACGCGGTGACTACAGGATATCTAATCC
TGTGTTCCTCCACACGTTCNTCNACACTCAGTCAGTATACGTACCAGGTGGCTCCTTC
GCCACTGGGTTTCTTCTATATCTACGCAATTTACGCTCAGACAGAAATTTACGCC
ACCCTCTCATTACATCTAGCTGCGCAGTTTGGAGTGACGTCTCCACAGGCAGGCCGG
GGCTTTTCAACTCCAATTTAACCAAGGCCACTTGCTTAGCTCACGCCGTTTACGCCG
TTCTGGAAAGTTCTCTGATGTCAAGGCCCCTGGAANGGTCTTCTCCTGCGTCGTCCG
ATATTCTGTCGGAACGNCAACNGCAAGGAATTAACCTTACTGCCCCTCTCC

RRB II-35
*Flavobacterium acidificum* strain LMG 8364 16S ribosomal RNA, partial sequence  
Sequence ID: NR_104962.1

*Pantoea agglomerans* strain DSM 3493 16S ribosomal RNA, partial sequence  
Sequence ID: NR_041978.1

*Pantoea conspicua* strain LMG 24534 16S ribosomal RNA, partial sequence  
Sequence ID: NR_116247.1

CGGCCCACTAGGCGANNTCCCTAGCTGCTGCTGAGGATGACCCNCCACACTTG
AACTGANAACACGGTCCANACTCTACTCAGGAGGCAGCTGGGGAATTATTCGACAAAT
GGGACGCAAGCCTGATGACCCATGCGGCTGTAATGANANGNNCTCAGGCTGNTGA
AGTACTCTCCAAGCAGGAGGATGATGNNNAATACCCGCGNNNGAGCATTTA
CCGCACNNNGAGCACCAGNTAAACTCCTGTCAGCCAGCCCGCGNNNTACGGAGGCT
GCAAACGCTTAATCGGAATTAGTTGGCGTTAACGGCGACAGCGGCGTCTTGCAAGTC
AGATGTCGAAATCCNNGGCTTAAAACCTGGAACCTGCAATTTGAACCTGCAAGGCT
AGTCGTGATAGGAGGGGTAGGATAATCAGATGCTGCTGAAATGCTGAGATCTCG
GAGGAAATACCGGTTGCGGAAGCGCGCCCGCTTGAGCACGAAGACTGACGCTGACGTG
CGAACAGCTGGGAGCAACAGGATATTAAATACGCTGTTAGCTCCAGCGGTAACAGAT
TGGCAGTTGGGCTTGCCTAGTGAGGAGTGGTCGCTCCGGGAAACCGTAAGACAG
GTTGCTGACATGCGTCTCCTCCTNNCTGGTTTNNNAAATGTTGGGTAAATGCTCAGGCAAC
AGCGCAACCCTATCTCCTNGTGGCCAGGGTCTTCGTGGGAACCTCAAAGGAANCTGC
CGGT
VITA

Ateet Maharjan was born in Chitwan, Nepal. He completed his undergraduate studies at Institute of Agriculture and Animal Sciences, Tribhuvan University with a B.S. in Agriculture. During his undergraduate practicum assessment, he worked on “Efficacy assessment of treatment methods against Powdery Mildew Disease of Pea (Pisum sativum L.) caused by Erysiphe pisi var. pisi.” After that, he worked as a Livelihood Officer in a Livelihood Project under World Vision for six months. In 2016 Fall, he started his graduate studies at Louisiana State University under the supervision of Dr. Jong Hyun Ham. He anticipates graduating with a master’s degree in Plant Pathology in August 2019.