Integrated management of sheath blight of rice by fertilizers, fungicides, and plant growth-promoting rhizobacteria

by

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Abstract

Sheath blight (ShB) is a soilborne disease causing major economic losses to rice cultivation. The disease is caused by a soil living basidiomycote fungal pathogen, *Rhizoctonia solani* Kuhn. Reliable and effective disease management strategies are needed for managing rice ShB disease. Most of the prevalent disease control methods are focused against the pathogen directly and have been moderately successful. Lack of durable sheath blight resistant rice varieties and environmental concerns about chemical usage have led to developing sustainable control methods using microorganisms. Advancements in biological control have led to identification and development of plant growth promoting rhizobacteria (PGPR) with plant and root growth stimulating ability. Besides, PGPR induce pathogen suppression by antagonism, competition for space and essential nutrients, and initiation of systemic resistance (ISR). The research objectives of this study were to 1) Screen strains of *Bacillus spp.* for biocontrol potential against multiple isolates of *Rhizoctonia solani* collected from diverse rice growing locations, 2) Evaluate combined efficacy of *Bacillus subtilis* (AP 301) and Azoxystrobin in managing rice sheath blight caused by *Rhizoctonia solani* under greenhouse conditions, and 3) Evaluate the combined effect of PGPR and fertilizers (NK) applied at different rates for the inhibition of sheath blight disease of rice under controlled environment.

In the first experiment, nine PGPR strains (AP 301, AP 52, AP 7, AP 136, AP 295, AP 305, AP 188, AP 294, and AP 209) were screened for *in vitro* antagonistic effect on *R. solani* and for *in vivo* plant growth promotion potential. Three *R. solani* isolates collected from Arkansas,

Mississippi, and Texas were used throughout the experiment. *In vitro* studies indicated that all the nine bacterial isolates inhibited *R. solani* mycelial growth by forming inhibition zones ranging from 0.3 to 4 mm. The most effective isolates were AP 301, AP 305, and AP 52 based on the *in vitro* mycelia and sclerotia inhibition tests against three isolates of *R. solani*. Nine bacterial strains were subjected to *in vitro* detached leaf assay. The majority of the strains had a lower percent lesion spread when compared with control. AP 301 isolate resulted in 65.72% lower *R. solani* lesion spread when compared to control. *In vivo* rice seed assay was performed for evaluating bacterial isolates' plant growth promoting properties. Isolates AP 294 and AP 301 showed increased plant growth by 26.5 and 25.5%, respectively, over control treatment.

The second study is to evaluate the combined efficacy of PGPR (AP 301) and fungicide against rice ShB. Strain AP 301 was evaluated at concentrations of 0, 10^3, 10^6, 10^9, 10^{11} CFU/ml (5 factors) in combination with azoxystrobin at 0, 396, 793, 1189, 1585, 1982, and at the recommended rate of 2,378 ppm (7 levels). Overall, azoxystrobin at the recommended rate (R), when used in conjunction with any of the concentrations of strain AP 301, resulted in complete reduction of ShB lesions (0% severity by RLH). Also, the results from other treatments tested suggest that combined application of *B. subtilis* AP 301 (at 10^9 CFU/ml) and Azoxystrobin @ 1189 of recommended rate) is an ideal dose of PGPR and fungicide in controlling ShB disease.

The last study is focused on PGPR and fertilizer compatibility applied for inhibition of rice ShB. PGPR at $1x10^9$ CFU/ml density was blended with different rates of nitrogen (N) and potassium (K) to evaluate their effect on ShB disease spread and their subsequent effect on rice yields. Pot culture experiments were treated with high, low, and recommended rates of fertilizers to determine the optimum dose of PGPR and fertilizer under controlled conditions. PGPR combined with N fertilizer applied at half the recommended rate resulted in lowest disease lesion

spread up to 2.83±0.15 mm and 2.33±0.16 mm for summer and fall experiments, respectively. For summer experiment, the treatment of PGPR combined with N applied at half recommended rate produced higher yields (23.12±0.33 g) than that of treatments applied using higher N rates. However, application of treatments consisting of PGPR and high rates of N fertilizer has produced lower yields ranging from 20.55±0.30 and 17.94±0.89 g. The experiment was repeated again in fall 2012, and our treatments showed a similar trend of disease and grain weight results.

In conclusion, PGPR, when applied with lower rates of N fertilizer, had significant effect on ShB disease spread in comparison to treatments with higher rates. PGPR in conjunction with different N fertilizer rates had a significant influence on rice grain yields.

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List of Abbreviations

PGPR	Plant Growth Promoting Rhizobacteria
IDM	Integrated Disease Management
ShB	Sheath Blight
RLH	Relative Lesion Height
ISR	Induced Systemic Resistance

Chapter I. Integrated disease management of rice sheath blight

Abstract

Rice is an important food grain and is a staple food for majority of the world's population. To meet increasing global demand and consumption, rice productivity must be enhanced. However, biotic stresses such as pests and diseases have impeded rice cultivation both in the tropics and subtropics. Of them, sheath blight is a major soil borne disease causing economic losses to rice cultivation. This chapter summarizes current information on the agronomic practices and biotic stresses affecting rice production and productivity. A detailed account is given of sheath blight (ShB) of rice, disease etiology and economics. Agronomic factors are important in ShB incidence and outbreak. Elaborative and updated accounts of various management options and their efficacy for ShB control are given. Specifically, the effects of popular cultural practices influencing ShB incidence, various chemical fungicides, and antibiotics individually and their combined effect on ShB are presented. Commonly used chemical fertilizers, especially nitrogen affect ShB incidence and intensity. The role of Plant Growth-Promoting Rhizobacteria (PGPR) and various genera of PGPR in ShB suppression are discussed. The present review also showed various aspects relating to ShB suppression by PGPR such as antagonism, competition for space and essential nutrients, and induction of systemic resistance. Results pertaining to effect of *Bacillus*, *Pseudomonas* and other important PGPR group on the causal agent of ShB, *Rhizoctonia solani* under laboratory, greenhouse and field conditions are included. Integrated management of ShB involving all the compatible combinations are included in this review with emphasis on use of PGPR as a main component.

Keywords: Rice, Sheath blight, Fertilizers, Antagonism, Biological control

I. Introduction

The world's population is expected to surge from 6.1 billion in 2000 to 9.2 billion in 2050 (UN, 2005). This prediction in human population requires increasing crop yields to meet the requirements of the rising global demand for food. At current annual rate, the world population is expected to grow at 1.2% or approximately 77 million people per year (Fernando, 2006). Six countries, India, China, Pakistan, Bangladesh, Nigeria, and Indonesia, account for majority of the annual population growth. Of these, four countries, India, China, Pakistan, and Bangladesh, are major consumers of rice cereal. Regardless of major advances in agriculture science over the past 50 years, a significant number of the world's population suffer from hunger and undernourishment. Lack of balance between crop production and demographic food demand is due to existence of hunger and malnutrition (FAO, 2002; Skamnioti and Gurr, 2009). With this population rise, it is expected that a corresponding food security problem will occur with the probability of losing agricultural land to industrialization and urbanization. Agriculture practices have incorporated greater part of world's available fertile farmland, which may limit further area expansion to low/non fertile land (Young, 1999; Cassman, 1999; Tilman et al., 2002). In addition to existing farmland problems, constrained farmland availability and identification of new plant diseases compound the challenges growers and scientists face globally to meet the nutritional requirements of the growing population. Globally, more than 3 billion people have rice as staple food, and it accounts for 50 to 80% of their daily calorie intake (Delseny et al., 2001). Over the next 20 years it is expected that demand for rice will grow by 2.5% per year (Hobbs, 2001). Ultimately, the challenge is to provide food for the increasing population and to ensure food security. To meet this demand the global rice production needs to be doubled by 2050 (Sheehy et al., 2008; Skamnioti and Gurr, 2009). Increasing rice yields is one of the few strategies to fight the world's food insecurity and malnutrition.

Rice is a monocotyledonous annual grass belongs to the family *Gramineae* and the genus *Oryza*. *Oryza* includes 20 wild species and two cultivated species: *Oryza sativa* (grown throughout the world) and *Oryza glaberrima* (grown only in Africa) (Pareja et al., 2011). Rice grain is a composite of carbohydrate, protein, fat, fiber, and other significant nutritive constituents (Torres-Escribano et al., 2008; Qian et al., 2010). Harvested rice is commonly known as rough or paddy rice. After the milling process, a final product is obtained that is white/milled/polished rice. Milling is a process where the outer layers of the grain is removed, exposing the white kernel inside. Rice comes in several sizes, colors, fragrances, and textures. Currently, China and India are ranked first and second in rice production according to Foreign Service Association of United States Department of Agriculture statistics (FSA/USDA, 2011). Together they account for 51.4% of total world milled rice production. Rice is known to have originated in China and have travelled to other parts of the world including the U.S.

Agriculture in the U.S. is diverse with the highest productivity when compared to other countries in terms of meeting its domestic food requirements and exporting a large amount of goods produced from a wide variety of crops (FSA/USDA, 2011). Rice is no exception. Rice production in the U.S. is known to be diverse and includes all type of rice: short, medium, long grain and specialty rice (FSA/USDA, 2011). Of the total estimate of world rice production of 450,200 thousand Metric tons (Mt), production in the U.S. accounts for up to 1.67% (FSA/USDA, 2011). A significant part of the rice produced in the U.S. is exported in the international market, ranking the U.S. third among the leading global exporters of milled rice (FSA/USDA, 2011). During the year 2009-2010, U.S. exports increased 15% due to tight global supplies and competitive prices (Baldwin and Childs, 2011). Each year in U.S., 2 million acres of cultivation produces 19 billion pounds of rice, out of which approximately 50% is supplied to the

domestic market (http://www.usarice.com/doclib/188/219/3674.PDF). Within the U.S., Arkansas, California, Louisiana, Mississippi, Missouri, and Texas are major rice contributing states (FSA/USDA, 2011). Planting, harvesting, and milling industrial advances have allowed the U.S. rice industry to flourish in a short time.

In the U.S., rice farm production is now a precise science with specialized equipment, lasers, and computers; there is no reliance on the seasonal rains as in Asian producing countries. Laser guided land leveling and recirculating irrigation systems allow farmers to increase yield and reduce the amount of crop water requirement. Growing domestic consumption and the opening of new international trade markets are expected to sustain the U.S. rice industry.

II. Agronomic practices for growing rice

Rice production may increase by one of several means or a combination such as enhancing production area and increasing crop yields through the use of chemicals, fertilizers, biological controls, and improved management of soil and water (Fernando, 2006). Also, using high yielding varieties, resistant cultivars, and promoting the use of genetically modified crops/cultivars resistant to pests and pathogens (Fernando, 2006). Rice production continues to progress due to intensified agronomy efforts and improved cultivation practices.

Appropriate agricultural practices need to be applied during rice production as they determine the level of grain production. The first step in rice production is choosing a rice seed with superior agronomic qualities and ensuring that the chosen seed suits the environment in which it will be grown. Quality and healthy rice seeds are prerequisite to good crop establishment under field conditions (McDonald, 1998; Phill, 1995; Farooq et al., 2011). Site selection is critical for rice production. Rice grows best in a warm climate with soil types which hold water well, preferably soils with high clay content to allow an underlain impervious claypan to maintain water standing. Before sowing, land is prepared and leveled by ploughing or

harrowing by using the correct tools or machinery available. Tillage followed by leveling reduces weeds, provides good crop establishment, and allows a uniform level of water standing during permanent flooding of rice fields.

Direct seeding and transplanting young rice seedlings are two popular methods of planting rice. Transplanting method is widely practiced across Asia. It involves transfer by hand or machine of young rice transplants from a seedling bed to a flooded field. Transplanting seedlings are raised in a nearby nursery where the seedlings are allowed to grow for 20-30 days, depending on the cultivar, before they are transplanted to the puddled or continuously flooded field. Hardened off seedlings are ideally transplanted on a warm day at ½ inch soil depth and 12x8 inches spacing with 2-3 plants per hill. Shallow planting is not beneficial as it may cause the seedlings to fall over. Deep planting should also be avoided as it results in slow development of tillers. The advantages of transplanting are increased nutrient availability (iron, zinc, and phosphorus) and inhibition of crop weeds (Surendra et al., 2001; Farooq et al., 2011). Direct seeding is defined as the process of establishing a rice crop from seeds sown in the field rather than by transplanting seedlings from the nursery (Farooq et al., 2011). Three main types of direct seeding of rice (DSR) are, dry seeding (sowing dry seeds into dry soil), wet seeding (sowing pre-germinated seeds on wet puddled soils), and water seeding (seeds sown into standing water) (Farooq et al., 2011). Advantages of DSR include saving time and labor. DSR is less popular than traditional transplanting because of poor crop stand, high weed infestation, and lower yields (Singh et al., 2005a). After rice seeding, proper irrigation, fertilizer management, and seasonal activities like weeding and controlling insect and pests are critical for rice growth and higher yields.

Rice cultivation is well suited to regions with high rainfall or fields under irrigated conditions, as it requires ample water to grow. Overall seasonal water input (rainfall and irrigation) to rice grown fields is 2-3 times higher when compared to other cereal crops (Tuong et al., 2005). Approximately 79 million ha of irrigated lowlands were used annually to produce up to 75% of world's rice production (Dawe, 2005 and Hafeez et al., 2007). Thus, rice is grown in a large area and requires a relatively large amount of water to grow. While various rice irrigation systems are accessible, the flooding method is popular with growers for its weed control. All other irrigation methods require extra cost and effort in checking weed population.

Globally, flooded rice fields produce greater e yield than rice grown in saturated conditions (Satyanarayana and Ghildyal, 1970; Grigg at al., 2000). In the U.S., rice is grown under flood throughout all or most of its development stage (Bollich et al., 1994; Grigg et al., 2000). Rice is well adapted morphologically and physiologically for surviving under water logged conditions for a long time. In flooded environments rice respires aerobically through facultative root arenchyma (Norman et al., 1995; McDonald et al., 2006).

Rice is a tropical crop, and strict irrigation management is followed based on crop growth stage requirement and climatic conditions. Rice is sensitive to drought during reproductive and ripening stages, and it is critical to increase the water level or create a permanent flood. Water acts as a dissolvent and carrier for many plant essential nutrients, and their uptake or loss is greatly influenced by efficient water management. The growth and final yield of rice yields are greatly affected by water and nutrients in the soil as well as their interaction (Benbi, 1989; Jin et al., 1999; Zhang et al., 2008).

The recommended rate of nitrogen (N), potassium (K), and phosphorus (P) is approximately 208-67-67 kg/ha (Groth and Bond, 2007). Potassium and phosphorus are usually

applied as single application before rice planting. The fertilizer rates may change with different ecologies of rice cultivation. Maintenance of suitable nitrogen application rates, plant spacing, shallow water depth and appropriate water management of rice fields are critical rice production management prerequisites to obtaining optimal grain yield (Lin et al., 2004; Lin et al., 2009). Nitrogen element is an essential plant nutrient and when applied at appropriate recommended rates is known to increase rice growth and yields. The type of N fertilizer applied to rice crop varies with dry seeding and transplanting methods.

Generally N applied on direct-seeded rice has more N use efficiency than a transplanted crop. Under flooded conditions, ammonium nitrogen (NH_4-N) is recommended because it is stable under flooded soil conditions. Usually, N is applied as a split application to deliver recommended rates during diverse crop growth phases. Plant isotopic N studies revealed that it recovers up to 70-75% urea-N when applied as three split applications (Snyder and Slaton, 2001). Three-way split application of total recommended N rate is usually applied as: to an estimated 50-70% is applied before planting at pre-flooding, 15 to 25% at ½ inch internode elongation and 15 to 25% at grain filling stage (Snyder and Slaton, 2001). Other macro nutrients and micronutrients are applied as per pre-soil test recommendations and upon observation of nutrient deficiency symptoms.

At commercial maturity rice is harvested either manually or using machinery, depending on the farm operation and size. Post-harvest management of rice using well-organized on-farm grain processing, storing, packing and transportation ensures that the rice quality is sustained for a long time. In spite of successful adaptation of recommended agronomic practices and establishment of rice crop, pests and pathogens are inevitable and protective methods should be available to minimize the crop loss.

III. Sheath blight of rice

The world's huge rice agro-ecosystem, designed to feed the ever increasing human population, also provides a habitat for great number of pests and pathogens. Rice diseases can cause significant quality and yield losses and can be a threat to the U.S. rice export industry. Rice sheath blight (ShB), caused by *Rhizoctonia solani* Kuhn (Teleomorph: *Thanatephorus cucumeris* (Frank) Donk) anastomosis group 1 IA (AG-1 IA), is a destructive disease worldwide that causes significant yield loss and quality degradation (Lee and Rush, 1983; Nagarajkumar et al., 2004). Apart from rice, the pathogen also infects many other plant species, including barley, lettuce, tomato, sorghum, and maize (Zhang et al., 2009). A significant amount of achievable rice production is safeguarded from *R. solani* by using protection strategies. In their absence, rice ShB disease causes 10-30% yield loss (Xie et al., 2008) and may reach up to 50% during prevalent years (Meng et al., 2001). In China only, about 15 to 20 million ha of rice growing area is affected, causing losses of 6 million tons of grains per year (Zhang et al., 2009). In Japan this disease can cause yield losses up to 20% and affects around 120,000-190,000 ha (Zhang et al., 2009). Planting ShB susceptible rice varieties in the U.S. resulted in yield losses of about 50% (Prasad and Eizenga, 2008; Zhang et al., 2009). In Arkansas, ShB was found present in 50-66% of rice fields, causing 5-15% yield losses in 2001 (Annou et al., 2005; Tan et al., 2007). The above findings indicate that ShB is a serious rice disease worldwide.

Rhizoctonia solani is a universal soil saprotrophic and facultative plant parasite (Ogoshi, 1996; Anees et al., 2010). It is an important soilborne plant pathogen and survives in soil by producing sclerotia (Baker and Martinson, 1972; Sumner, 1996; Keijer, 1996; Tsai et al., 2012). The species *R. solani* is actually a diverse fungal group and is divided using anastomosis grouping (AGs) based on anastomosis reactions of its hyphae (Sneh et al., 1991, Carling et al., 1999; Lübeck and Poulsen, 2001). The word 'Anastomosis' refers to the capacity of hyphae from

two different isolates to fuse. The AG groups are further subdivided into AG 1- AG 4 and AG 6- AG 9 based on fungal characteristics like morphology, virulence, host range, nutritional requirements, DNA sequences, and biochemical and molecular properties (Carling et al., 2002; Anees et al., 2010).

Rhizoctonia solani has limited movement due to lack of spores and survives in unfavorable conditions by forming sclerotia or dormant mycelia (Sumner, 1996; Anees et al., 2010). Sclerotia in soil can survive for 2 years, and are spread during field preparation and flooding the field for irrigation (Webster and Gunnell, 1992; Brooks, 2007). During permanent flooding the sclerotia may float and move within the field or to bordering fields through continuous flood irrigation. Sclerotia or hyphae attach to the plant, infecting and causing ShB disease, and the pathogen spreads under conditions favorable to disease development. Soilborne pathogens normally are dormant and immobile in the field, so the host plant typically grows towards the stationary pathogen (Gilligan, 1983; Anees et al., 2010). With pathogen and plant contact, mature sclerotia cause ShB infection. Initial symptoms occur on leaf sheaths near the water line as water-soaked lesions. Secondary infections are caused by hyphae growing upward towards uninfected plant parts, producing additional lesions and sclerotia on leaf sheaths to complete the disease cycle (Webster and Gunnell, 1992; Brooks, 2007).

The disease peaks during flowering when the rice canopy is most dense, forming a microclimate favorable to pathogen growth and spread (Brooks, 2007). *R. solani* can infect seed to fully mature plant, causing moderate to significant yield losses depending on the plant part affected. Visible plant disease symptoms include formation of lesions, plant lodging, and presence of empty grains. Large lesions formed on infected sheaths of lower rice leaves may lead to softness of the stem thereby initiating stem lodging (Wu et al., 2012). Lodging alters the

normal rice canopy design, affecting photosynthetic ability and total biomass production (Hitaka, 1969; Wu et al., 2012). ShB presence during flowering or panicle initiation causes a reduction of total seed weight due to a lower percentage of filled spikelets and results in significant yield losses (Cu et al., 1996; Nagarajkumar et al., 2004). During rice sheath blight epidemics, severe lodging may occur, which obstructs the transportation of water, nutrients, and carbohydrate assimilates through the xylem and phloem channels, affecting grain filling (Kashiwagi et al., 2005; Wu et al., 2012). Disease spread and intensity is dependent on the amount of infectious inoculum present in planting material and residues of previous crop remaining in the field or in the top soil where rice is grown. Other impact factors for ShB disease severity are rice development stage at infection, ecological surroundings, cultivar resistance, and cultural and seasonal crop practices (Gangopadhyay and Chakrabarti, 1982; Groth et al., 1992). The presence of one or many factors may enhance the severity of ShB beyond economic threshold levels, thereby incurring low to high yield losses.

IV. Key agronomic factors aggravating rice Sheath blight disease

The use of high rates of N fertilizer, double cropping, high plant densities, and early maturing, dwarf build, high tillering and susceptible varieties have been shown to enhance ShB severity in most of the world's rice growing areas (Lee and Rush, 1983; Nagarajkumar et al., 2004). In addition, plant morphological traits of rice cultivars are known to enhance ShB severity. Planting of semi-dwarf cultivars with more tillers caused serious rice ShB in southern China (Liao et al., 1997; Zuo et al., 2009). Studies conducted on relationship between plant morphological characters and their relation with ShB development confirmed that plant height alters microclimate and light transmission inside the dense canopy, thereby facilitating disease development (Han et al., 2003; Tang et al., 2007).

Role of fertilizers in rice ShB disease

It is common for growers to apply rates of N higher than those recommended for rice. This results in lush green vegetative growth conducive to pathogen spread. Application of excess N is not only hazardous to the soil and water environments but also increases rice ShB severity. Rice produces increased numbers of tillers when high N doses are applied, which increases its susceptibility to pathogens and insects. Rice ShB disease intensity and incidence increase with increased rates of N fertilizer application (Groth and Bollich, 2000). High N rates also facilitate the ShB disease development and spread by increasing tiller density and moisture retention inside the rice canopy (Savary et al., 1995; Tang et al., 2007). Therefore, careful N fertilizer management should be adopted for rice crop, which would increase nitrogen use efficiency during the growing season and diminish ShB disease concerns.

Potassium (K) is another major essential element which has a role in ShB disease etiology due to its involvement in resistance mechanisms of the host plant (Cakmak, 2005; Li et al., 2010). However, the mechanism by which K stimulates resistance towards a pathogen is not completely understood (Amtmann et al., 2008). Dordas (2008) reported K-deficient plants are highly susceptible to disease due to alteration of metabolic functions of K in plant physiology. The accepted explanation for the mechanism by which K enhances plant resistance to pathogens is from the mechanical resistance point of view. This involves development of thicker cell wall of epidermal cells in the presence of K (Mengel et al., 2001; Li et al., 2010). Experiments conducted on maize stock rot disease showed that K-applied plant cells have abundant golgi bodies, which could produce a high amount of secretions to degrade the mycelia (Li et al., 2010).

V. Sheath blight disease management

Rice plants respond to various stresses in their surroundings by comprising attack by pests and pathogens like bacteria, fungi, virus, and nematodes. Plant defense responses

correspond to the type of attacking external agent. The plant capacity to respond to an infection is determined by both the host and pathogen genetic traits (Gullino et al., 2000). To protect against pathogen infection plants have conferred various defense mechanisms such as, gene-for-gene interactions, systemic acquired resistance (SAR), and signal transduction networks that require jasmonic acid and ethylene (Glazebrook, 2001; Ramonell and Somerville, 2002). However, a virulent pathogen overcomes a plant's defense mechanism by evading the effects of activated defenses, avoiding triggering plant defenses, or suppressing the plant's resistance reactions (van Loon et al., 1998). The virulent pathogen that escapes from the plant's natural defense will cause disease on parts. A range of ShB disease control measures aimed at protecting growers, millers, and other allied businesses have been reported in the literature.

A. Cultural control

One approach to sustainable disease management without the use of chemicals is to develop disease resistant cultivars. Benefits from disease resistant cultivars include low cost of fertilizers, reduced disease incidence and increased grain and milling yields (Groth et al., 1993). To date, resistance breeding efforts against ShB has been only moderately successful, mainly due to a lack of source for resistance in cultivated rice or in wild related species (Pan et al., 1999; Brooks, 2007). Nevertheless, rice cultivars ranging from susceptible to moderately resistant to ShB are available for cultivation. Cultivars exhibiting partial ShB resistance include jasmine 85, Teqing, LSBR-5, and YSBR1 (Wang et al., 2011). These moderately resistant cultivars show low to moderate infection without the use of other disease management strategies.

Developing a new rice breeding line consisting of high and stable ShB resistance along with superior agronomic traits is challenging. To gain a better understanding of how plants respond to pathogen challenge is to uncover important plant immunity regulatory networks and determine how they interfere with basic pathogen processes like growth, metabolism, and

development. Using plant breeding programs, researchers manipulate the identified pathogen resistant genes to develop commercially resistant cultivar. However, the development of new resistant cultivars was hampered through direct screening of germplasm because the fungal pathogen *R. solani* is plurivorous and semisaprobiotic (Zou et al., 2009). Developing a new line of ShB disease resistance was a primary goal of rice breeding and plant pathology researchers in the southern U.S. (Groth and Bond, 2007). Current lack of effective resistant cultivars has led growers to rely increasingly on chemical fungicides.

B. Chemical control

Fungicides are widely used for combating rice ShB infection. At this time there is no rice cultivar that is highly resistant to ShB (Anees et al., 2010). However, fungicides remain essential for successful management of rice ShB disease after the pathogen has infected young plants. Scouting rice fields to estimate ShB disease intensity is usually recommended before any fungicide application. Scouting is usually performed from the stages of internode elongation to heading. Fields are scouted randomly to identify positive disease symptoms. Fungicide application is recommended when 5-10% positive disease areas are recognized in the scouted field. Both systemic and non-systemic fungicides are now available, with the former having more market space and value. Since systemic fungicide introduction in the 1960s, they have gradually replaced the non-systemic products, establishing better disease management and opening new fungicide markets (Gullino et al., 2000).

Each year new products are added to the market through fungicide research by agrichemical companies. Current fungicide research is mainly aimed at identification of suitable and novel target sites (Gullino et al., 2000). Several fungicides having novel modes and sites of action are available to growers for suppression o *R. solani*. Several rice ShB fungicides representing different chemical groups are available, including inorganic (Copper and sulfur),

organic (carbamates, dicarboximides, etc.), systemic (propiconazole, strobulin, carboxamides), Quinone outside inhibitors [QOLs], and benzimidazoles, antibiotics, and fumigants. Within each group there may be multiple labeled fungicides sold under different brand/trade names worldwide. Carboxamide/oxathiins fungicides are sold as boscalid, carboxin, benodanil, furametpyr, penthiopyrad, flutolanil, mepronil, fenburam, and thifluzamide (Zhang et al., 2009). The modes and sites of action differ with each chemical group of fungicides. For example, carboxamides fungicides are single-site inhibitors of the succinate ubiquinone reductase or succinate dehydrogenase complex in the respiratory chain (FRAC, 2007; Zhang et al., 2009). They are effective seed treatments for control of *R. solani*. At present, systemic fungicides belonging to the strobilurin group are used extensively to combat rice ShB pathogen.

Difficulties in extensive production of natural chemical compounds, and their relative volatility and light instability are the reasons for not applying them directly in field conditions (Gullino et al., 2000). Optimization of natural compounds has led to the development of fungicides such as, azoxystrobin (Azn) and kresoxim-methyl (Sauter et al., 1996). These fungicides have broad spectrum activity against fungi. Originally, this group of fungicides was termed as β-methoxyacrylates due to their common structure to β-methoxyacrylate moiety early analogues (Clough, 1993; Clough et al. 1995; Gullino et al., 2000). However, different molecules with similar mode of action targeting the site bc_1 complex were identified, and they are structurally related to strobilurin (Gullino et al., 2000). Hence, these compounds are named as strobilurin class (Sauter et al., 1996).

Within the strobilurins group, azoxystrobin fungicide is widely used as it works effectively against ShB pathogen infestation (Groth and Bond 2006). The fungicide is a derivative of β-methoxyacrylate and was the first registered fungicide from this class of

chemistry (Anonymous, 1996; Grichar et al., 2004). Azoxystrobin is a diverse group of fungicide and used as eradicant and protectant on both plant (foliar and seed) and soils (Godwin et al., 1992; Gullino et al., 2000). Azoxystrobin can be applied as either foliar spray or granular form to control rice ShB (Gullino et al., 2000). Azoxystrobin is sold as Quadris 2.08 SC (chemical product of Syngenta, Raleigh, NC). The mode of action of azoxystrobin is to inhibit electron transport at a unique site of action and kill fungal pathogens (Anonymous, 1996; Grichar et al., 2004). It can be used as a preventative measure in minimizing the spread of disease or as a systemic fungicide to check the development of disease (Grichar et al., 2000; Grichar et al., 2004). Preliminary studies reported by Groth et al. (1993) suggest that optimum application time of azoxystrobin for effective control of ShB changes with disease epidemics during the growing season. Fungicide rate and composition vary with intensity of disease and the type of cultivars (susceptible/medium susceptible/ moderately resistant) used. The common recommended dosage of azoxystrobin is 0.17 kg a.i. ha^{-1} at the heading initiation stage (Anonymous, 1999; Groth, 2008). Benefits from this fungicide include lower disease incidence, likely reduction of inoculum, and improved grain and milling yields (Groth, 1996; Groth and Bond, 2006).

Azoxystrobin is considered the best fungicide in the U.S. for sheath blight control (Groth, 2008). However, it can be expensive and ineffective if applied under low disease incidence (Groth et al., 1992; Groth 2005; Groth 2008). In commercial rice fields under light to moderate ShB disease conditions, fungicide applications are not recommended (Groth et al., 1992). The fungicide is mostly applied as a single application, and two applications are recommended under severe ShB disease conditions (Groth, 2008). However, current economic and environmental constraints limit the second fungicide application. Groth (2008) reported that single application

of azoxystrobin has similar responses to two applications on different rice ShB susceptible cultivars.

Research findings suggest the chance of a pathogen developing resistance to a particular chemical increases with regular use over a period of time (Brent and Hollomon, 1998; Zhang et al., 2009). The alternatives are to develop a new line of a chemical (fungicide) class that has no cross resistance to the chemical to which the pathogen developed resistance originally or to develop other preventative strategies free of chemicals. Manufacturing and releasing a new line of pesticide into the market are time consuming, expensive, and involve the risk of failure.

Recently it was reported that only few chemicals have been introduced thereby creating shortage of pathogen control tools for most specialty crops (Zilberman and Millock, 1997). One reason for this may be due to stringent pesticide regulations adopted by policy makers to prevent the use of chemicals considered too dangerous to human health and the environment. Chemical control, though effective in managing disease often has a significant impact on humans and the natural environment through the pollution of soils, above and below ground water resources, and the entire food supply chain. Human health and environmental protection regulations are strict. A major goal in developing a new fungicide is to ensure a good balance between potency and safety (Knight et al., 1997). A fungicide that is effective against disease but fails to meet the area/topic regulations standardized by the representative group/organization may be banned completely from use. For example, the European Union has banned the usage of the ShB fungicides validamycin and jinggangmycin due to their potentially harmful effects on health and ecological surroundings (Commission of the European Communities, 2002). Furthermore, new pesticide should undergo constant reassessment, re-registration and changing guidelines of application techniques and residue levels (Zilberman and Millock, 1997).

A product that clears all regulations is patented and sold in the market. Patent time varies with the country of application. The success of the product is not guaranteed, as it may have competition from rival products, and it may develop pathogen resistance. To ensure continued efficacy of fungicides appropriate management strategies are required (Urech et al., 1997). Despite these efforts, pathogen resistance is still a limiting factor in potential use of fungicides in crop protection (Gullino et al., 2000). Each time a pathogen develops resistance to a pesticide in use, a replacement for the existing pesticide should be readily available. Due to the uncertainty of pathogen behavior to chemicals, it is necessary to develop non-chemical control methods.

C. Biological control of ShB

Lack of durable sheath blight resistant rice varieties and environmental concerns about chemical usage have led to developing sustainable control methods using microorganisms. Antagonism between organisms is common in the ecosystem and is most prevalent among soil microorganisms. Natural interference between beneficial soil microorganisms and plant pathogens results in zone of buffer, thereby inhibiting or reducing disease development (Köhl et al., 2011). Simply, this natural buffering is the result of biological control of unwanted microorganisms by other competing plant or soil microorganisms (Köhl et al., 2011). Various microbial defense mechanisms may work independently or together, depending on the rhizosphere or phyllosphere characteristics.

Microbial antagonistic properties have created new opportunities in biological control technology. The first step in this process is to isolate and identify the role of antagonistic microorganisms responsible for biological control. The next step is to multiply potential antagonists in the laboratory and test them under lab, greenhouse, and field conditions. Before marketing, field efficacy of the final product is tested at multilocation sites covering various related crops (same family taxa). Market introduction of new products are known as plant

protectants in most countries and similar chemical protectant regulations and protocols apply (Köhl et al., 2011).

Several biological control methods have been proposed to control ShB; however, their usage by farmers has been limited due to elusive application techniques and the inconsistency of field effects (Xie et al., 2008). Managing soil-abundant beneficial microbes for the improvement of plant root and shoot growth and plant health is an exciting field. Complex interactions of soil-plant-microbes can impact plant vigor and yield (Kennedy, 1998). These interactions in the rhizosphere also influence plant health and soil fertility (Jeffries et al., 2003). Rhizosphere microbial interaction benefit plants by increasing soil available crop nutrients (Dey et al., 2004). Advancements in biological control have led to identification and development of antagonistic bacteria with plant and root growth stimulating ability.

Plant growth- promoting rhizobacteria

Rhizosphere isolated, free living soil bacteria with proven plant beneficial properties are known as plant growth- promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). Besides, PGPR role in increasing plant or root growth, they directly influence increased N uptake, phosphate solubilization, phytohormone synthesis, and production of iron chelating siderophores (Lalande et al., 1989; Bowen and Rovira, 1999; Wu et al., 2012). Some PGPR are used commercially to enhance plant growth and health. For example, PGPR formulations for seed, soil, and spray treatments (leaves and fruits) have been developed. Seed treatment of rice with PGPR resulted in increased root and shoot length of seedlings (Kumar KVK, et al. 2009). PGPR beneficial effects have been reported in wide range of crops (Dubey, 1996).

Based on PGPR relationship with plants, they are known as symbiotic bacteria and free living rhizobacteria (Khan, 2005). PGPR are also known for biological control of various soil-inhabiting bacteria. PGPR are used in ecofriendly products. They are naturally available in the

environment and provide resistance against a broad spectrum of pathogens (Radjacommare et al., 2004). The microbial populations in rhizosphere can be influenced by soil characteristics, agronomic practices, and plant type (Radjacommare et al., 2004). Inconsistent results of PGPR applications between the laboratory, greenhouse, and field studies can be due to changes in climate or soil (Lucy et al., 2004). An improved understanding of microbial population dynamics is needed before amending the farming practices to enhance plant growth and yield.

PGPR induce pathogen suppression by different modes of action such as antagonism, competition for space and essential nutrients, and initiation of systemic resistance (ISR) (Wu et al., 2012). The concept of activating plants defense pathways to control pathogen infection is appealing, though difficult to implement effectively. Induced resistance occurs when a plant, once appropriately stimulated, exhibits an enhanced resistance upon challenge inoculation with the pathogen (Dutta et al., 2008). This type of resistance is mostly systemic in nature, spreading from point of infection to other distant plant parts (Dutta et al., 2008).

Seed treatment with some PGPR strains induced ISR in treated plants (Kloepper et al., 1999; Wu et al., 2012). Various bacterial determinants are claimed to elicit ISR. For example, fluorescent pseudomonads produce siderophores, antibiotics (Persello-Cartieaux et al., 2003) and *Bacillus* strains produce cyclic peptides and aminopolyols, which triggers plant ISR (Yu et al., 2002).

Although PGPR are grouped as various bacterial taxa, genus *Pseudomonas* and *Bacillus* are commercially more exploited (Kumar KVK, et al. 2009). Genus *Bacillus* is considered to be one of the most diverse and comprehensively studied PGPR group (Garbeva et al., 2003; Beneduzi et al., 2008). Several *Bacillus* and *Paenibacillus* were commercially exploited for developing common plant growth promoters and biological fungicides, insecticides, nematicides

(Beneduzi et al., 2008). In addition, some *Bacillu*s spp. have shown increased plant growth and yield (Pal and Jalali, 1998; Ponmurugan and Shyamkumar, 2011). *Bacillus* spp. are spore forming, gram-positive, rod shaped bacteria which are highly tolerant to adverse environmental conditions (Kokalis-Burelle et al., 2006). The resistant endospores of *Bacillus* spp. provide toleration to pH extremes, pesticides, fertilizers, and heavy metals (Ponmurugan and Shyamkumar, 2011). Endospore formation also confers bacterial stability during formulation and storage of products, thereby making it a valuable commercial bacterial inoculant (Kokalis-Burelle et al., 2006). PGPR might be more effective when combined with other ShB disease control methods through an integrated approach.

D. Integrated disease management

In many countries rice is grown in the same season and the same field year after year, making it more susceptible to soilborne pathogens. Over time, pathogen inoculum accumulates in crop soil or surrounding fields and can cause epiphytotic disease. Over use or over dependence on chemical control or any other single control method is not sufficient to manage rice ShB. A systemic control approach uniting all ShB disease management options may produce better pathogen management. Integrated disease management (IDM) of rice ShB is broad-based, ecological plant pathogen control approach, combining all the available disease control methods with each method compensating the deficiencies of others (Kumar KVK, et al. 2009). It reduces the emphasis on fungicides by including other disease control methods. IDM is an environmentally- sensitive approach and is gaining popularity worldwide. IDM is recommended year round to monitor major crop programs. A monitoring-based IDM program helps enhance the pathogen control and reduces environmental quality related to fungicide crop inputs (McDougall et al., 2002). Water quality becomes impaired when fungicides and sediments move off-site and into nearby water streams or leach into soil and contaminate ground water.

Environmental measures to minimize water and air quality problems using an IDM program should be considered for all pesticide or fungicide applications. This program in general covers all major pests and pathogens of rice. However, a suitable IDM program assembling all ShB disease control practices can be developed and used for profitable rice farming.

The main goal of an IDM program is to inhibit plant diseases from occurring by using resources with minimal environmental concerns. Planning, selecting ShB management tactics, considering field, environmental, and economic aspects, and evaluation are vital before IDM implementation. Every agronomic or horticultural commodity has associated pest and disease, and field scouting efforts are instrumental in identifying potential problem (ShB). Planning is the starting step towards a successful IDM program. Before selecting the appropriate rice cultivar to grow, one should study the cropping system, disease history, pathogen lifecycle, and rotational plan for the chosen field. Collecting random soil samples in the field for nutrient, salinity, and pH analysis helps to determine field suitability and soil nutrient management for rice crop. Also, the soil should be sampled for potential disease-causing nematodes and soilborne pests and pathogens and treated as per IDM guidelines (Parker et al., 2002). Ponding water in rice fields suppresses most weeds population. However, regular surveying and managing weeds are required to avoid crop weed competition for nutrients, light, and space and to avoid weed harboring of potential disease- causing pests.

Following field selection, it is necessary to prepare the field based on the soil test results. Cultural practices such as determining bed size, preparing soil bed, choosing an irrigation system, selecting a planting configuration (number of seedlings, spacing etc.), eliminating weed and alternate host (if any), and determining recommended fertilizer applications are important considerations during field preparation. When land preparation is finished, the appropriate

cultivar should be selected by considering the time and season of year in which it is to be grown and the range of ShB disease resistance. After planting, fertilizers should be applied based on prior soil sample test results. Monitoring pathogen or pathogen damage (disease symptoms) throughout crop growth until harvest is important to determine which management practices is needed. Ideal IDM program integrates all disease management options such as chemical, cultural (sanitation, crop rotation), biological, mechanical (tillage, radiation, or heating soil), and legal (quarantine.) control methods. Fungicide compatibility with IDM programs is very important for sustainable agriculture. Chemicals with low toxicity towards beneficial organisms and non-target species will have a strong competitive advantage over products with lower standards concerning human and environmental health (Groth, 2008). Chemical control should be regarded as a last resort. Despite efforts to avoid using chemicals, there are times when only fungicides can control the damage. Therefore, economic factors must be considered because fungicides should be used in an IDM program only when the benefits (yield and quality) exceed the cost of chemical control. Finally, growers should evaluate the IDM program by monitoring and record keeping. IDM data recording for crop yields and control measures act as a guide if the same problem occurs. Evaluation of IDM in terms of effectiveness by comparison with other control program's benefits results in a successful adoption program. Educating farmers and disseminating information about effective and environmentally-sound IDM mitigate rice ShB pathogen damage while accomplishing sustainable farming.

VI. References

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Chapter II. Evaluating strains of Bacilli for plant growth promotion and biocontrol potential against *Rhizoctonia solani* on rice (*Oryza sativa*)

Abstract

Sheath blight (ShB) of rice causes significant economic and agricultural grain loss. Reliable and effective disease management strategies are needed for managing rice ShB disease caused by *Rhizoctonia solani* (*R. solani*). Selection of best plant growth-promoting rhizobacteria (PGPR) strains is a vital step in ShB disease management. From a total of 70 bacterial strains, nine isolates exhibiting antagonistic effects against *R. solani* in preliminary tests were selected for further screening. In this experiment, nine PGPR strains (AP 301, AP 52, AP 7, AP 136, AP 295, AP 305, AP 188, AP 294, and AP 209) were screened for *in vitro* antagonistic effect on *R. solani* and for *in vivo* plant growth promotion potential. Three *R. solani* isolates collected from Arkansas, Mississippi, and Texas were used throughout the experiment. *In vitro* studies indicated that all the nine bacterial isolates inhibited *R. solani* mycelial growth by forming inhibition zones ranging from 0.3 to 4 mm. The most effective isolates were AP 301, AP 305, and AP 52 based on the *in vitro* mycelia and sclerotia inhibition tests against three isolates of *R. solani*. Nine bacterial strains were subjected to *in vitro* detached leaf assay. The majority of the strains had a lower percent lesion spread when compared with control. AP 301 isolate resulted in 65.72% lower *R. solani* lesion spread when compared to control. *In vivo* rice seed assay was performed for evaluating bacterial isolates' plant growth promoting properties. Isolates AP 294 and AP 301 showed increased plant growth by 26.5 and 25.5%, respectively, over control treatment. These results suggest that the bacterial isolates AP 301, AP 305, and AP 52 have superior *in vitro* antagonistic properties against *R. solani* and significant *in vivo* plant growth promoting ability on rice seeds.

I. Introduction

Sheath blight (ShB) disease of rice is a destructive fungal disease worldwide and results in severe economic yield losses to the growers if not managed appropriately. The soil-borne fungus *Rhizoctonia solani* (*R. solani*) is the causal organism of ShB. The pathogen causes yield losses of up to 30% and reduces rice quality (Xie et al., 2008). Infection occurs when pathogen inoculum surviving in the soil comes in contact with a rice plant under conditions favorable to disease development. Infectious mycelium from previous crop debris and dormant sclerotia in the soil are two main sources of pathogen survival (Kozaka, 1961; Kobayashi et al., 1997). In rice fields the pathogen survives mainly as sclerotia. These are infectious fruiting bodies formed due to aggregation of vegetative mycelium. Sclerotia may be brown initially but over time turn to a darker brown color. Sclerotia are irregularly shaped, small to large in size, immature to mature (age of sclerotia), and able to survive in soil or residual plant debris for many years. After flooding rice fields, floating sclerotia come in contact with rice leaves/sheaths and infects the succeeding transplanted crop (Kotamraju, 2010). Early ShB symptoms are seen near lower end of rice shoots where floating sclerotia contact seedling and infection progress upwards. During severe ShB disease incidence lesions on leaves coalesce and may result in lodged rice fields. Severely affected plants may have unfilled rice grains (Kotamraju, 2010).

A multitude of disease management approaches have been evaluated against ShB pathogen and had moderate success. At this time there is no rice cultivar that is highly resistant to ShB (Anees et al., 2010). Chemical control of rice ShB is currently widespread. However, chemical control of ShB is expensive and non-sustainable. Also, intensive use of fungicides places huge selection pressure on pathogen and thereby possibly develops fungicide resistance (Brent and Hollomon, 1998; Zhang et al., 2009). These concerns have prompted research in

pathogen control that seeks an integrated biological control approaches which are durable and sustainable.

Ecofriendly trials that determine plant beneficial soil microorganisms have gained importance for ShB management. Among these organisms, the most widely studied group by plant pathologists is plant growth promoting rhizobacteria (PGPR). In addition to their plant growth promoting properties they are well known phytopathogen antagonists. Several phytopathogenic (Fungal, bacterial and viral) diseases under greenhouse and fields conditions were effectively managed using PGPR mediated induced systemic resistance (ISR) (Kloepper et al., 2004; Zhang et al., 2010). In planta ISR determination is activated by pathogen attack and is followed by systemic production of several defense compounds (Dean and Kuc, 1985; Senthilraja et al., 2013). These compounds suppress pathogen invasion by mechanisms like cytolysis, leakage of potassium ions, and inhibition of mycelial growth and protein biosynthesis (Quan et al., 2010; Laslo et al., 2012).

Beneficial bacterial populations belonging to various genera thrive in diverse rice ecosystem. However, their population size depends on seed source, seed quality, seed health, and whether the seed sown was germinated or pre-germinated (Cottyn et al., 2001). Genus *Bacillus* is considered to be one of the most diverse and comprehensively studied PGPR group (Garbeva et al., 2003; Beneduzi et al., 2008). Several *Bacillus* and *Paenibacillus* were commercially exploited for developing common plant growth promoters and biological fungicides, insecticides, nematicides (Beneduzi et al., 2008). In addition, some *Bacillu*s spp. have shown increased plant growth and yield (Pal and Jalali, 1998; Ponmurugan and Shyamkumar, 2011). Application of bacilli strains along with other disease control methods in an integrated practice in rice field may be a long-term, sustainable alternative to ShB control.

Currently, PGPR application in the field is at a rudimentary stage. With extensive positive research results, it has the potential to be economically marketed as a tactic for ShB pathogen management. Hence, in this research, we performed various laboratory and greenhouse studies to evaluate PGPR strains that are antagonistic and possess plant growth- promoting properties.

II. Materials and Methods

Culture media and organisms used for experiment

A virulent *R. solani* inoculum isolated from field grown rice (cv. Cocodrie) infested with ShB disease was used for this research. The infectious sclerotia were collected by Dr. Shane Zhou (Assistant professor at Texas A&M University System, AgriLife Research & Extension Center, Beaumont, TX, USA). The pathogen was maintained on potato dextrose agar (PDA) prior to use.

All experimental treatments of PGPR strains were obtained from Dr. J. W. Kloepper laboratory culture collection (Professor at Department of Entomology and Plant Pathology, Auburn University, AL, USA). Before using PGPR for lab studies, strains were tested for purity as suggested by Kotamraju, 2010. The PGPR stored in tryptic soy broth (TSB) culture amended with 20% glycerol at -80^0 C were obtained. The bacterial cell suspensions were streaked on tryptic soy agar (TSA) plates and incubated for one day to check the purity. After 24 h of incubation, the bacterial cells or colonies were harvested from TSA plates in sterile distilled water and centrifuged for 5 min at 6000 rpm (Raj et al., 2012). The bacterial pellets were resuspended in sterile water and spectrophotometer was used to adjust PGPR treatments concentration or density to desired levels. The above purity check for PGPR treatments was performed during each assay and before they were applied as treatments.

Source of rice cultivar

Seeds of rice cultivar Cocodrie were obtained from Louisiana State University, research center located at Crowley, LS were used for this study. Rice seeds were stored under cool temperature

conditions (4-5^0 C) before seeding. Cocodrie cultivar was successful in USA due to its early seed development and superior grain yielding ability. However, Cocodrie rice cultivar is susceptible to ShB disease causing huge economic yield losses.

***Rhizoctonia solani* mycelial growth inhibition assay**

Mycelial growth inhibition assay was performed to evaluate the efficacy of PGPR strains against the three isolates of *R. solani* collected from Arkansas (AK), Mississippi (MS), and Texas (TX). Freshly prepared and sterilized TSA plates (size, 100 x 15 mm) were used to conduct the assay. This assay was performed as suggested by Kotamraju, 2010. Using a cork (size 2) an active fungal mycelium growing on PDA was transferred on to the center of round TSA plate. PGPR treatments were streaked as uniform lines (3.5 cm) on either side of mycelium plug. Plates were sealed and randomly placed in an incubator for 5 days. In total there were ten treatments which include nine PGPR strains and a healthy control. Treatments were repeated five times to reduce experimental variability. Plates were transferred to an incubator maintained at a temperature of 27^0 C. The plates were later observed for treatment effects on mycelium spread and zone of inhibition. A formula suggested by Gupta et al., 2001, was used to calculate the percentage of mycelia inhibition.

$$I(\%) = \frac{100(C - T)}{C}$$

Alphabets in above formula represent, I = Percentage inhibition of *R. solani* mycelial growth, C = *R. solani* mycelial growth in the control plate (mm), and T = Mycelium growth of *R. solani* in plates treated with PGPR (mm).

***Rhizoctonia solani* sclerotia growth inhibition assay**

Sclerotia growth inhibition assay was performed as suggested by Kazempour, 2004. Freshly grown (10 day old) *R. solani* isolates (AK, MS, and TX) sclerotia on PDA culture plates were

isolated in to three separate labeled sterilized petri plates. Prior to use, pathogen inoculum was disinfected by pouring 20 ml sodium hypochlorite (2.5% concentration) in to a sclerotia holding plate and allowed it to stay for 2 minutes. After 2 min residue sclerotia were thoroughly rinsed with sterile distilled water to wash off the chemical. Three sclerotia growth inhibition assays were conducted, each assay represent separate pathogen isolate. PGPR treatments were grown in a 250 ml conical flasks filled with 50 ml TSB. Flasks were then placed on a rotary shaker at room temperature for incubation and the speed and time were adjusted to 175 rpm and 24 h, respectively. After 24 h, five disinfected sclerotia where carefully transferred in to each flask. Using shaker with similar settings the flasks containing PGPR and sclerotia culture was mixed thoroughly. Using a forceps the sclerotia were carefully removed from flask and placed on PDA plates. Each plate consist four sclerotia placed on four sides of quadrant position. Plates were sealed with a tape and randomly placed in Precision Gravity Convection incubator set at 27^0C for 5 days. Overall there were 10 treatments for each assay and each treatment was replicated five times. Plates were later observed for treatment effects on sclerotia inhibition. A formula suggested by Gupta et al., 2001, was used to calculate the percentage of mycelia inhibition.

$$\mathrm{I(\%)} = \frac{\mathrm{100(C-T)}}{\mathrm{C}}$$

Alphabets in above formula represent, I = Percentage inhibition of *R. solani* mycelial growth, C = mycelial growth of sclerotia in the control plate (mm), and T = Mycelium growth of sclerotia in plates treated with PGPR (mm).

In vitro detached leaf assay

There are two steps involved in this assay. The first step is growing rice seedlings (cv. Cocodrie) in pots under greenhouse conditions as suggested by Kotamraju, 2010. Clean 0.5 gallon artificial pots were filled to top with field soil. The soil type is loamy to sandy and was obtained from Lee

County, Alabama. Before seeding, pots were watered to breakdown the large soil aggregates. Once the soil is settled uniformly inside the pot, three seeds were sown per pot. After 15 days of rice seed emergence they were thinned back to one seedling per pot. Pots were arranged in a randomized complete block design and the seedlings were allowed to grow for 60 days inside the non-chemical zone greenhouse chamber. The crop growing conditions were replicated inside the greenhouse by adjusting the temperature, relative humidity, and light quality to 26 ± 2^{0} C 90±1%, and 16 h per day, respectively.

The second step involves conducting Guleria et al., 2007 suggested detached leaf assay method on leaves of 60 day old seedlings. After performing purity check, using a Turner spectrophotometer the treatments (PGPR) density was adjusted to $4x10^{8}$ CFU ml^{-1}. Freshly harvested rice leaves were transferred from greenhouse to laboratory in a plastic storage box. The box was covered on all sides with dampened tissues to retain the leaf moisture content during transport. Leaves were later sterilized using 1.5 % sodium hypochlorite for 2 min and sized to 8 cm. One cut leaf was placed on a dampened filter paper fitted in to a 14 cm diameter sterile petri plates. Mature 10 day old sclerotium was placed in the middle of detached leaf and thick disinfected glass slips were placed on the edges to prevent leaf movement. Finally, the treatments were sprayed covering the entire surface of leaf. This step was repeated for each treatment per each replication per each assay. Each treatment was replicated five times to minimize the experimental error or variability. Later the petri plates were transferred in to growth chamber and incubated for 7 days. The growth chamber temperature, relative humidity, and light were adjusted to 26 ± 2^{0} C 90±1%, and 16 h per day. After incubation the plates were retrieved and observations were made for sheath blight lesion spread near the sclerotium. Relative lesion lengths (RLH) were calculated using a formula suggested by Sharma et al., 1990.

$$\text{RLH (\%)} = \frac{100(\text{Total lesion heights})}{\text{Total height of leaf}}$$

In vivo rice seed growth assay

Sterilized rice seeds (cv. Cocodrie) were used in rice seed growth assay. PGPR culture solution was prepared at a density of $1x10^9$ CFU ml^{-1} and used as rice seed treatment before seeding. This step was performed separately for each of nine experimental PGPR strains. PGPR treated and untreated (control) seeds were soaked in wet cheese cloth at room temperature for three days before being transferred to pots. Three seeds were planted in 0.5 gallon pots consisting of field soil obtained from Lee County, Alabama. Soil type was loamy to light clay with CEC = 4.6-9.0 $cmolckg^{-1}$. Plants were incubated in the greenhouse (chemical free zone) at a temperature of 26 ± 2^0 C, 90±1% moisture content, and light adjusted to 16 h per day. Overall, there were 50 pots consisting of nine treatments and five pots per treatment. Rice seedling lengths (shoot and root) and percent vigor index were measured after 20 days by randomly picking five seedlings from each treatment. The formula used was (Kotamraju, 2010):

$$Seed\ vigor\ index = Germination\ percent * Plant\ growth\ (Shoot + root\ lenghts)$$

Statistical analysis

The data were analyzed using SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). All experimental data from this chapter were analyzed by restricted maximum likelihood (REML) approach using Proc-Mixed analysis. The treatment averages were differentiated by Tukey's least significant difference test at probability level P=0.05. Pearson correlation coefficient analysis was performed comparing the potential of PGPR against three isolates of *R. solani*. Proc-Correlation was used for correlation analysis of variables.

III. Results

Rhizoctonia solani mycelial growth inhibition assay

For this experiment**,** bacterial treatments had a significant difference ($P<0.05$) from control on mycelial growth of *R. solani* (3 isolates) after 7 days. Three isolates were analyzed separately for percentage inhibition of mycelial growth. Total means of percent of mycelial inhibition for the AK, MS, and TX isolates were 56.8 ± 0.96, 56.6 ± 0.99, and 62.0± 0.52, respectively (Tables 1 through 3). In AK experiment, three antagonists, AP 301, AP52, and AP 188, showed maximum suppression of mycelial growth on PDA. However, bacterial isolates of AP294 and AP7 had lower mycelial inhibition than the average total means (< 56.8 ± 0.96) (Table 1). The range of percent inhibition of AK mycelia was from 0 to 71%, and the inhibition zones ranged from 0 to 3.5 mm (Table 1).

In the experiment with MS, four bacterial isolates, AP 301, AP52, AP305, and AP 188, showed over 60% mycelial inhibition (Table 2). Antagonist *Bacillus safensis* (AP7) had the lowest percentage inhibition of 34.2 ± 1.11 (Table 2). The range of percent inhibition of MS mycelia was from 0 to 69%, and the inhibition zones ranged from 0 to 4 mm (Table 2). Similarly, four antagonists, AP 301, AP305, AP 188, and AP52, had over 63% mycelial inhibition in the TX pathogen isolate experiment. The highest and lowest percent inhibition numbers were 70.4 ± 0.50 and 58.8 ± 0.73 of bacterial isolates AP 301 and AP7, respectively. Inhibition zones ranged from 0 to 3.3%. Pearson Correlation Coefficients test was performed to evaluate the linear association among the three pathogen isolates. When compared to each other, a significant positive correlation existed between three *R. solani* isolates obtained from AK, MS, and TX (Table 4 and Figure 1).

Rhizoctonia solani sclerotia growth inhibition assay

Isolates showing antagonistic effects on mycelial inhibition were screened for suppression of *R. solani* sclerotia. In this study, treatments had a significant effect ($P<0.05$) on inhibition of sclerotial germination over control. Total means of percent of sclerotial inhibition for the AK, MS, and TX isolates were 68.9 ± 3.93, 69.4 ± 5.01, and 69.0± 6.13, respectively (Table 5). In AK experiment, four bacterial isolates, AP 301, AP 52, AP 305, and AP 188, had over 90% sclerotial inhibition (Table 5). Bacterial antagonist isolates AP 52, AP 301, and AP 305 had 90% sclerotial inhibition of fungal pathogen isolates from MS and TX (Table 5). The lowest inhibition for AK, MS, and TX was observed with sclerotia treated with isolate AP 7 (Table 5).

In vitro detached leaf assay

All nine PGPR isolates tested had significant effect on lowering ShB lesion size when compared to the control (Table 6). The total means of percent of sclerotial inhibition for the AK, MS, and TX isolates were 24.7 ± 0.69, 26.3 ± 0.46, and 26.3 ± 0.56, respectively (Table 6). Application of PGPR strains from AK, MS, and TX resulted in disease severity ranging from 10.8% to 34.2%, 9.6% to 36%, and 12.6% to 35.6%, respectively (Table 6). Among the PGPR strains tested against 3 pathogen isolates, lowest ShB lesion development was obtained with *B. subtilis* AP 301 with 9.6% of lesion spread (Table 6). Isolate AP 301 had up to 65.8% lower lesion spread when compared with control treatment against *R. solani* isolate MS. Other significant lesion reduction of ShB was noticed with *B. subtilis subsp. subtilis* strains AP 52, AP 305, and AP 188 with less than 22% lesion spread (Table 6).

In vivo rice seed growth assay

In vivo rice seed growth assay was evaluated under greenhouse conditions for measuring plant growth ability of PGPR treated seedlings (Table 7). Root and shoot lengths and seedling vigor index was measured to evaluate bacterial strains' potential. PGPR treatments had significant

effect on treated plants with a P value of 0.003. In the presence of treatments, the highest seedling vigor was found for isolate AP 305, which enhanced plant growth by 29.24% compared with the control. When compared to control treatment, isolates AP 294 and AP 301 increased the rice seedling vigor by 26.5% and 25.4%, respectively.

IV. Discussion

Rhizobacteria are well known for plant growth promotion and suppression of soil borne pathogens (Hoflich et al., 1994; Weller, 1988; Rajkumar et al., 2004). Screening studies in our lab performed by Dr. Krishna Kumar (Dr. Klopper's lab, Plant pathology, Auburn University) resulted in the selection of nine efficient strains from approximately 70 strains isolated from the rhizosphere. The objective of this investigation was to select the most efficient PGPR antagonists against infection of *R. solani* isolates collected from AK, MS, and TX in the USA. Nine strains were subjected to series of screening tests to select the best strain for rice ShB management. The conventional *in vitro* screening assay was performed to evaluate ShB antagonistic bacterial strains. Of the nine isolates, AP 301, AP 52, and AP305 produced measurable zones of inhibition against *R. solani* isolates from AK, MS, and TX. Large inhibition zones ranging from 3 to 3.83 mm were present after 5 days of incubation. The above three antagonists consistently produced over 60-71% mycelial inhibition against AK, MS, and TX pathogen isolates (Tables 1 through 3). Previous research indicated that PGPR isolates produced β 1, 3-glucanase, salicylic acid, and HCN for inhibition of *R. solani* mycelium (Nagarajkumar et al., 2004; Kotamraju, 2010). Figure 1 scatter plots show that the prediction ellipses range from 70 to 80% for three pathogen isolates when treatments were applied. Nearly circular ellipses indicate the strong linear association among the three pathogen isolates.

Germination of sclerotia from *R. solani* isolates was drastically reduced by most *Bacilli* strains (Tables 1 through 3). Isolates AP 301, AP52, and AP 305 had 90% sclerotial inhibition against all tested pathogen isolates (Table 5). This suggests the presence of fungistatic metabolites and antibiotics secreted by *R. solani* isolates. PGPR has been reported to decrease *R. solani* sclerotial growth and cause cell lysis (Kazempour, 2007; Kotamraju, 2010). Cell suspensions of PGPR treated sclerotia for 1 min to 4 weeks resulted in inactivation (Pande and Chaube, 2003; Kotamraju, 2010). Further, the nine isolates were screened *in vitro* by detached leaf assay to resemble field conditions where all the components (pathogen, antagonist, and host plant) of research interest interact (Rajkumar et al., 2004). This assay allows consistent and reproducible inoculation of *R. solani,* resulting in realistic measurement of lesion spread (Kotamraju, 2010).

Leaves were selected for screening as they are more prone to ShB disease attack than other rice plant parts. Since *R. solani* is soilborne and infection spread through foliage, foliar application of PGPR is important for ShB management (Kotamraju, 2010). Isolates AP 301 and AP 52 had the lowest sheath blight lesion spread, when compared to other treatments. PGPR produced defense compounds like phenylalanine ammonia-lyase, peroxidases, chitinases, glucanases, thaumatin-like proteins, and PR proteins may inhibit ShB severity (Jayaraj et al., 2004; Kotamraju, 2010). However, lesion spread in some isolates (AP 136, AP294, and AP 295) was high or close to maximum spread compared to that observed on control. The reasons for this may be the slow establishment of antagonistic bacteria on detached rice leaves. Some antagonistic bacteria require an initial period of time to survive on the leaf before suppressing growth of the pathogen (Rajkumar et al., 2004).

Furthermore, the same isolates were tested *in vivo* for plant growth enhancement. In this assay, most PGPR treatments increased shoot length and root length of rice seedlings when compared to that of control. Isolates AP 305, AP 294, and AP 301 had higher seedling vigor index when compared with other treatments. PGPR are well known for their plant growth promotion properties (Kloepper et al., 1980). When compared with control there was up to 30% increase of rice seed vigor treated with PGPR. Application of PGPR inoculants improved rice nutrient uptake, growth, seedling vigor and yield (Biswas et al., 2000; Chithrashree et al., 2011).

A correlation was plotted to estimate the direct proportionality of shoot and root growth for treated rice seedlings (Figure 2). A positive correlation value of 0.71 indicates the shoot growth is dependent on the size of the rice roots. These experiments showed that majority of tested PGPR strains were successful in vegetative inhibition of *R. solani* and besides their crop growth improvement properties.

V. References

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VI. Appendix

Table 1. Effect of treatments on *in vitro* mycelial growth of *Rhizoctonia solani* isolate from Arkansas

			***R. solani* isolate (Arkansas)**	
Treatment no.	**Strain**	**Identification**	**% Inhibition of mycelial growth[1]**	**Inhibition zone[2] (mm)**
T1	AP 301	*Bacillus subtilis*	70.80±0.37[a]	3.33[a]
T2	AP52	*Bacillus subtilis subsp. subtilis*	63.60±1.28[b]	3.27[a]
T3	AP7	*Bacillus safensis*	33.40±1.24[f]	0.36[c]
T4	AP136	*Bacillus amyloliquefaciens*	57.60±1.66[d]	2.17[b]
T5	AP295	*Bacillus amyloliquefaciens*	57.20±1.02[d]	2.11[b]
T6	AP305	*Bacillus amyloliquefaciens subsp. plantarum*	61.80±0.97[bc]	3.1[ab]
T7	AP188	*Bacillus amyloliquefaciens*	62.40±1.36[bc]	3.23[a]
T8	AP294	*Paenibacillus peoriae*	45.20±2.44[e]	0.76[c]
T9	AP209	*Bacillus mojavensis*	59.20±1.53[d]	2.46[ab]
T10	Control		----	----
Total mean			56.80±0.96	

[1]Mycelial growth was recorded at 5 days after incubation
[2]Width of inhibition zone between pathogen and PGPR was measured at 5 days after incubation
Results are mean averages of 5 replicates
Means or Means ± standard error followed by the same letter are not significantly different according to Tukey's multiple range test at P = 0.05

Table 2. Effect of treatments on *in vitro* mycelial growth of *Rhizoctonia solani* isolate from Mississippi

			***R. solani* isolate (Mississippi)**	
Treatment no.	**Strain**	**Identification**	**% Inhibition of mycelial growth[1]**	**Inhibition zone[2] (mm)**
T1	AP 301	*Bacillus subtilis*	69.20±0.73[a]	3.83[a]
T2	AP52	*Bacillus subtilis subsp. subtilis*	65.20±1.16[b]	3.27[ab]
T3	AP7	*Bacillus safensis*	34.20±1.1[f]	0.73[c]
T4	AP136	*Bacillus amyloliquefaciens*	57.00±0.71[d]	1.92[bc]
T5	AP295	*Bacillus amyloliquefaciens*	57.80±1.71[d]	2.23[b]
T6	AP305	*Bacillus amyloliquefaciens subsp. plantarum*	64.40±0.50[b]	3.15[ab]
T7	AP188	*Bacillus amyloliquefaciens*	62.20±2.08[bc]	2.93[ab]
T8	AP294	*Paenibacillus peoriae*	58.80±1.77[cd]	2.27[b]
T9	AP209	*Bacillus mojavensis*	40.40±0.81[e]	0.84[c]
T10	Control		---	---
Total mean			56.57±0.99	

[1]Mycelial growth was recorded at 5 days after incubation
[2]Width of inhibition zone between pathogen and PGPR was measured at 5 days after incubation
Results are mean averages of 5 replicates
Means or Means ± standard error followed by the same letter are not significantly different according to Tukey's multiple range test at P = 0.05

Table 3. Effect of treatments on *in vitro* mycelial growth of *Rhizoctonia solani* isolate from Texas

			***R. solani* (isolate - Texas)**	
Treatment no.	**Strain**	**Identification**	**% Inhibition of mycelial growth[1]**	**Inhibition zone[2] (mm)**
T1	AP 301	*Bacillus subtilis*	70.40±0.50[a]	3.23[a]
T2	AP52	*Bacillus subtilis subsp. subtilis*	63.80±0.37[b]	2.93[a]
T3	AP7	*Bacillus safensis*	58.80±0.73[cd]	1.55[c]
T4	AP136	*Bacillus amyloliquefaciens*	59.00±0.89[cd]	1.63[c]
T5	AP295	*Bacillus amyloliquefaciens*	58.20±0.86[de]	1.83[bc]
T6	AP305	*Bacillus amyloliquefaciens subsp. plantarum*	65.80±1.07[b]	3.29[a]
T7	AP188	*Bacillus amyloliquefaciens*	64.80±0.58[b]	3.14[a]
T8	AP294	*Paenibacillus peoriae*	56.20±0.37[e]	1.43[c]
T9	AP209	*Bacillus mojavensis*	60.60±0.92[c]	2.22[b]
T10	Control		---	---
Total mean			61.96±0.52	

[1]Mycelial growth was recorded at 5 days after incubation
[2]Width of inhibition zone between pathogen and PGPR was measured at 5 days after incubation
Results are mean averages of 5 replicates
Means or Means ± standard error followed by the same letter are not significantly different according to Tukey's multiple range test at P = 0.05

Table 4. Correlation values of *in vitro* mycelial growth between *Rhizoctonia solani* isolates collected from Arkansas (AK), Mississippi (MS), and Texas (TX)

Pearson Correlation Coefficients, N = 45 **Prob > |r| under H0: Rho=0**			
	AK	**MS**	**TX**
AK	1	0.6905 <.0001	0.67642 <.0001
MS	0.6905 <.0001	1	0.52843 0.0002
TX	0.67642 <.0001	0.52843 0.0002	1

Boxed values include correlation and P value for each isolate when compared to others. Plotted against same isolate it is perfectly correlated with a value of 1(diagonal).

Table 5. Effect of treatments on percent sclerotia inhibition of *Rhizoctonia solani* isolates collected from Arkansas, Mississippi, and Texas.

% Inhibition of Sclerotial germination (*R. solani* isolates)					
Trt no.	**Strain**	**Identification**	**Arkansas**	**Mississippi**	**Texas**
T1	AP 301	*Bacillus subtilis*	100±0.00^{a}	92±4.89^{a}	96±4.00^{a}
T2	AP52	*Bacillus subtilis subsp. subtilis*	96±4.00^{ab}	96±4.00^{a}	92±4.89^{a}
T3	AP7	*Bacillus safensis*	32±4.89^{d}	16±7.48^{d}	24±11.66^{c}
T4	AP136	*Bacillus amyloliquefaciens*	40±6.32^{d}	60±10.95^{bc}	52±10.19^{bc}
T5	AP295	*Bacillus amyloliquefaciens*	72±8.00^{bc}	80±12.64^{ab}	80±8.94^{ab}
T6	AP305	*Bacillus amyloliquefaciens subsp. plantarum*	92±4.89^{ab}	92±4.89^{a}	92±4.89^{a}
T7	AP188	*Bacillus amyloliquefaciens*	92±4.89^{ab}	84±7.48^{ab}	88±4.89^{ab}
T8	AP294	*Paenibacillus peoriae*	52±4.89^{cd}	68±8.00^{ab}	60±12.64^{abc}
T9	AP209	*Bacillus mojavensis*	44±7.48^{d}	36±4.00^{cd}	36±9.79^{c}
T10	Control		---	---	---
	Total Avg. Mean		68.88±3.93	69.33±5.01	68.9±6.13

Percentage sclerotial inhibition was recorded at 5 days after incubation
Results are an average means of 5 replicates
Means or Means ± standard error followed by the same letter are not significantly different according to Tukey's multiple range test at P = 0.05

Table 6. Efficacy of PGPR treatments on ShB lesion suppression in a detached leaf assay

***R. solani* isolates % Sheath blight lesion spread[1](mm)**					
Trt. no.	**Strain**	**Identification**	**Arkansas**	**Mississippi**	**Texas**
T1	AP 301	*Bacillus subtilis*	10.14±0.79^{d}	8.60±0.33^{d}	11.60±0.46^{d}
T2	AP52	*Bacillus subtilis subsp. subtilis*	15.80±0.64^{c}	16.80±0.65^{c}	18.40±0.45^{c}
T3	AP7	*Bacillus safensis*	27.60±1.83^{a}	31.30±0.70^{a}	30.40±1.07^{ab}
T4	AP136	*Bacillus amyloliquefaciens*	29.80±0.88^{a}	29.60±0.98^{a}	31.20±1.08^{ab}
T5	AP295	*Bacillus amyloliquefaciens*	30.20±0.25^{a}	31.40±0.46^{a}	29.60±0.59^{ab}
T6	AP305	*Bacillus amyloliquefaciens subsp. plantarum*	20.80±0.14^{b}	18.80±0.26^{bc}	17.60±0.29^{c}
T7	AP188	*Bacillus amyloliquefaciens*	19.60±0.84^{bc}	20.40±0.81^{b}	20.80±1.06^{c}
T8	AP294	*Paenibacillus peoriae*	29.60±0.34^{a}	31.60±0.61^{a}	31.80±0.88^{ab}
T9	AP209	*Bacillus mojavensis*	28.80±0.76^{a}	29.80±0.82^{a}	28.60±0.75^{b}
T10	Control		31.40±1.01^{a}	32.60±0.62^{a}	32.60±0.34^{a}
	Total mean		24.37±0.75	25.09±0.62	25.26±0.70

[1]Sheath blight lesion spread was recorded at 7 days after incubation by Relative Lesion Height method.
Results are an average mans of 5 replicates
Means or Means ± standard error followed by the same letter are not significantly different according to Tukey's multiple range test at P = 0.05

Table 7. *In vivo* experiment showing PGPR strains influence on rice seedling shoot length, root length, and seedling vigor index.

			Plant growth[1]		
Trt. no.	**Strain**	**Identification**	**Shoot length (cm)**	**Root length (cm)**	**Seedling vigor index**
T1	AP 301	*Bacillus subtilis*	24.68±1.72	9.02±0.91	3370±152
T2	AP52	*Bacillus subtilis subsp. subtilis*	23.98±1.90	8.42±0.94	3240±284
T3	AP7	*Bacillus safensis*	20.98±2.40	6.1±0.55	2708±75
T4	AP136	*Bacillus amyloliquefaciens*	22.74±1.57	6.48±1.44	2922±196
T5	AP295	*Bacillus amyloliquefaciens*	24.30±0.90	7.96±0.77	3226±169
T6	AP305	*Bacillus amyloliquefaciens subsp. plantarum*	24.74±1.65	10±0.65	3474±168
T7	AP188	*Bacillus amyloliquefaciens*	20.82±3.58	8.59±1.16	2856±658
T8	AP294	*Paenibacillus peoriae*	24.70±1.21	9.30±0.22	3400±66
T9	AP209	*Bacillus mojavensis*	22.44±0.57	6.74±0.98	2918±243
T10	Control		21.00±3.68	5.90±1.58	2688±481

[1]Shoot and root lengths were measured 20 days after seeding in pot.
Results are an average means of 5 replicates ± standard error.

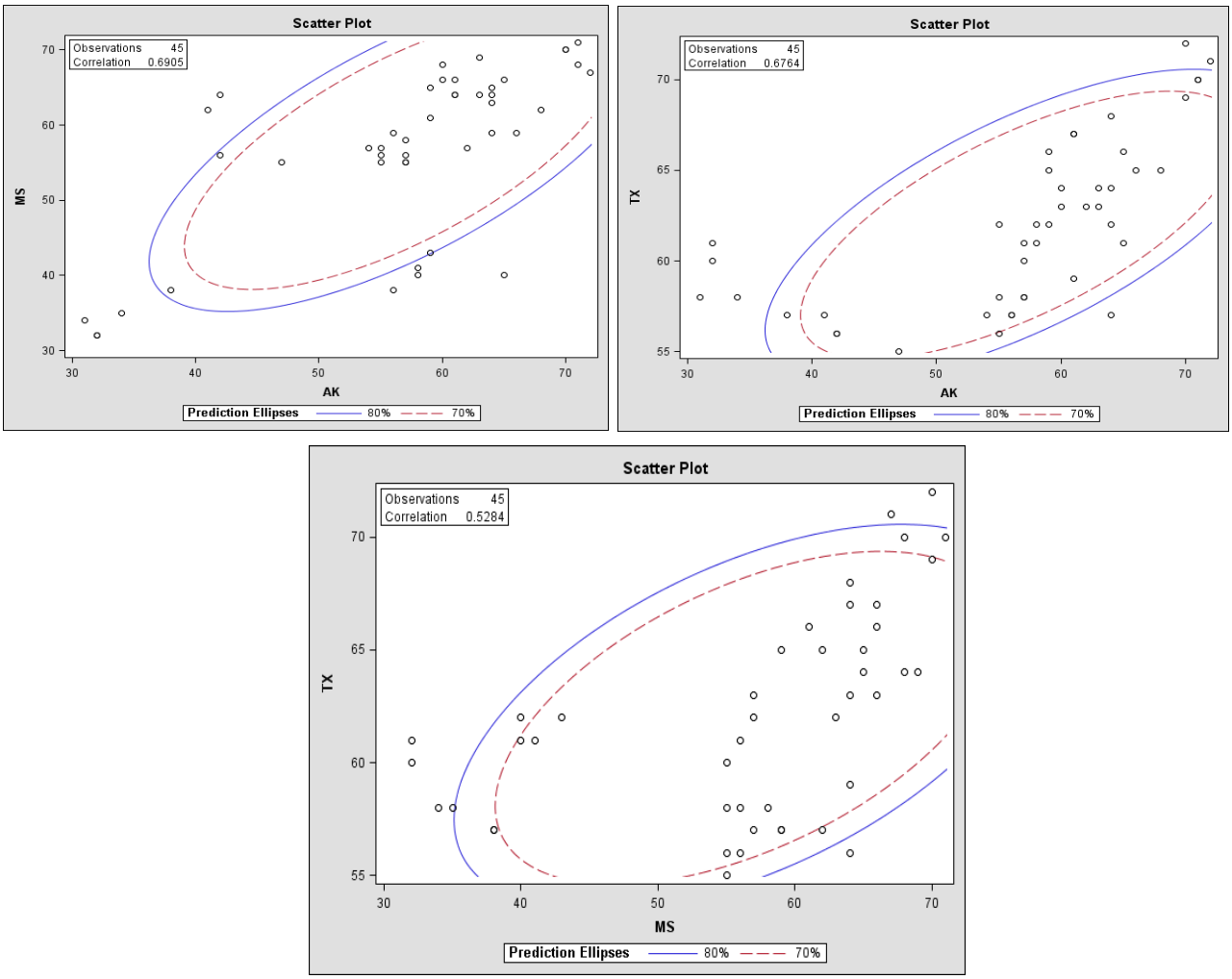

Figure 1. Correlation scatter plots of *in vitro* mycelial growth between *Rhizoctonia solani* isolates collected from Arkansas (AK), Mississippi (MS), and Texas (TX)

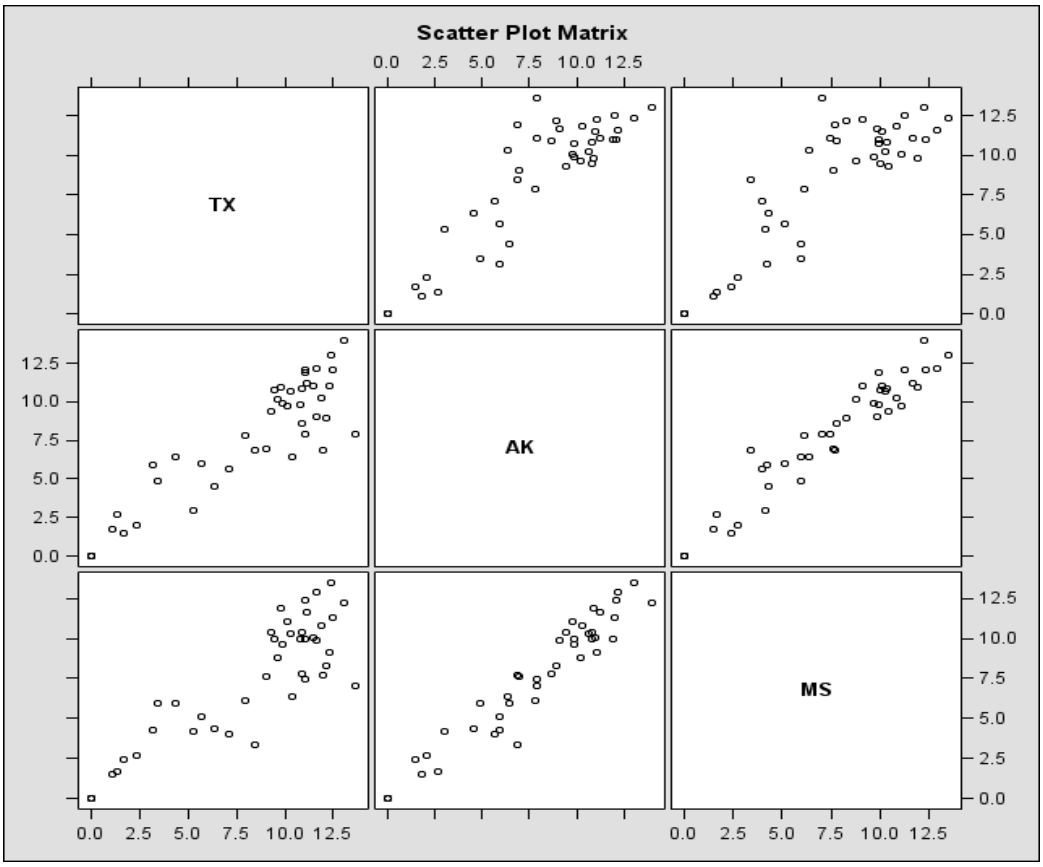

Figure 2. Correlation scatter plot showing the root and shoot lengths are dependent on each other when treated with PGPR strains.

Chapter III. *In vivo* screening of PGPR isolates for biological control of *Rhizoctonia solani* and compatibility with fungicides

Abstract

Among various biotic stresses affecting rice, sheath blight (ShB) is a significant fungal disease causing economic crop losses. The disease is caused by a soil living basidiomycote fungal pathogen, *Rhizoctonia solani* Kuhn. Screening and selection of elite plant growth-promoting rhizobacteria (PGPR) strains and their compatibility with conventional fungicides are vital in developing ShB integrated disease management at field level. One research objective was to screen in the greenhouse nine strains (AP 301, AP 52, AP 7, AP 136, AP 295, AP 305, AP 188, AP 294, and AP 209) for inhibition of *R. solani* mycelial growth. The best strain (AP 301) from these results was chosen for further fungicide compatibility testing. Two greenhouse experiments were conducted at the Plant Science Research Center, Auburn University, Auburn, AL, USA. Three *R. solani* isolates collected from Arkansas (AK), Mississippi (MS), and Texas (TX) were used for screening experiment. Only the TX isolate was used for fungicide compatibility experiment. The identified best nine strains, along with healthy control and pathogen control treatments, were tested for suppression of ShB disease on rice in terms of relative lesion spread and disease severity. Highest reduction in disease lesion spread was obtained when seedlings inoculated with AK, MS, and TX isolates were sprayed with strain AP 301. Compared to pathogen control, when sprayed with AP301 lesion spread on plants was reduced by 84.47%, 83.82%, and 86.54% for AK, MS, and TX, respectively. The second experiment evaluated the combined efficacy of AP 301 and Azoxystrobin fungicide against rice ShB by using 5 x 7 factorial randomized complete block design (RCBD). Strain AP 301 was evaluated at concentrations of 0, 10^3, 10^6, 10^9, 10^{11} CFU/ml (5 factors) in combination with azoxystrobin at 0, 396, 793, 1189, 1585, 1982, and at the recommended rate of 2,378 ppm (7

levels). Overall, azoxystrobin at the recommended rate (R), when used in conjunction with any of the concentrations of strain AP 301, resulted in complete reduction of ShB lesions (0% severity by RLH). Also, the results from other treatments tested suggest that combined application of *B. subtilis* AP 301 (at 10^9 CFU/ml) and Azoxystrobin @ 1189 of recommended rate) is an ideal dose of PGPR and fungicide in controlling ShB disease.

I. Introduction

Among all cereal crops grown worldwide, rice (*Oryza sativa* L.) is one of the major food crops. Rice production levels are declining as rice is prone to infection from pathogens which cause mild to destructive damage. Sheath blight (ShB) disease is destructive in most major rice growing regions of the world (Kotamraju, 2010). ShB is caused by a soil living basidiomycote fungal pathogen, *Rhizoctonia solani* Kuhn (*R. solani*). During offseason, pathogen survives as sclerotia or mycelia, or both, in soil/plant debris and on weeds in rice growing regions (Kobayashi et al., 1997; Krishna Kumar et al., 2012). Sclerotium is considered to be the primary inoculum that overwinters and infects the subsequent rice crop. Under conditions favorable to disease development, such as low light intensity, high humidity (>95%), and temperature (28-32°C) in crop microclimate canopy, ShB spreads rapidly through mycelium in all directions (Su et al., 2012). Infection by *R. solani* may result in lodging of rice, reduced yields, poor grain quality, and soil infestation with pathogen inoculum. Minimizing the incidence of ShB disease epidemics and decreasing yearly crop losses are essential to sustain rice production (Mew et al., 2004; Chithrashree et al., 2011).

Use of control measures is central to combating ShB disease. At this time only susceptible and moderately susceptible to moderately resistant rice cultivars are available for ShB pathogen. About 90% of the rice cultivation area in the U.S. is planted with susceptible cultivars (Parissa and Tarighi, 2011; Su et al., 2012). The pathogen is known to overcome plant

resistance through the emergence of novel fungal strains, which has made developing a new fully resistant cultivar difficult (Lucas et al., 2009). Rice ShB deterrent product market is currently dominated by chemical compounds. Chemical control of ShB is most effective when applied during early stages of rice ShB detection. However, fungicidal treatment of ShB is often inconsistent as there is a chance of the pathogen developing resistance against the chemicals. In addition, fungicides also have a negative impact on air, soil, and water ecosystems. Protecting these ecosystems is a priority by many countries. Due to heightened environmental concerns of agricultural production methods in U.S., various chemicals were banned permanently from use. In 2005, under the Montreal protocol, methyl bromide was completely banned in the U.S. due to its ozone depleting effects (Carter et al., 2005). The harsh consequences of this ban have led manufacturers and growers to consider an integrated approach to managing sheath blight disease. The focus or emphasis of integrated diseases management (IDM) is to lower agricultural chemical input by incorporating other feasible cultural and biological control practices in agriculture production.

Biological control involves the use of biological processes and products as well as organisms (micro and macro) for disease control (Cook, 1993; Wilson, 1997). Products and organisms used in biological control are commonly referred to as biological control agents. Rise in plant growth promoting rhizobacteria (PGPR) usage as a biocontrol agent in managing rice diseases and increasing crop yields has been reported (Mew and Rosales, 1992; Krishna Kumar et al., 2012). PGPR are naturally occurring, free living, soil dwelling, and root inhabiting bacteria that have beneficial effects on soil and plants. Plant growth can be influenced directly or indirectly through PGPR application. As indicated, PGPR have a direct effect on plant growth promotion. Some of the direct plant benefits due to the addition of PGPR are increased seed

germination rates, root growth, yield, leaf area, chlorophyll content, magnesium content, nitrogen, protein content, hydraulic activity, tolerance to drought, shoot and root weights, and delayed leaf senescence (Lucy et al., 2004).

PGPR are known to cause disease suppression by antagonism, competition for space and nutrients, and triggering plant inducted systemic resistance (ISR) (Kumari and Srivastava, 1999; Chithrashree et al., 2011). A variety of factors related to biotic and abiotic components and chemical elicitors trigger ISR, which in turn may activate plant resistance to invading pathogens (Chester 1933; Ross 1966; Hull 2001; Cassells and Rafferty-McArdle, 2012). A single strain or a combination of several PGPR strains may confer plant resistance to pathogens. PGPR-mediated ISR by the same strain can suppress a broad spectrum of plant pathogens (Zhang et al., 2002). In certain plant diseases, compatible PGPR mixtures give better protection over the use of an individual PGPR strain (Jetiyanon and Kloepper, 2002). Therefore, continuous application of PGPR to rice fields is effective against ShB on a long term basis and can be an alternative or supplement to fungicides.

Several pathogen antagonistic species belonging to various genera thrive in rice ecosystem. Genus *Bacillus* is considered to be one of the most diverse and comprehensively studied PGPR group (Garbeva et al., 2003; Beneduzi et al., 2008). Several *Bacillus* and *Paenibacillus* are commercially exploited for developing common plant growth promoters and biological fungicides, insecticides, nematicides (Beneduzi et al., 2008). In addition, certain *Bacillu*s spp. have shown increased plant growth and yield (Pal and Jalali, 1998; Ponmurugan and Shyamkumar, 2011). *Bacillus* spp. are spore forming, gram-positive, rod shaped bacteria which are highly tolerant to adverse environmental conditions (Kokalis-Burelle et al., 2006). The resistant endospores of *Bacillus* spp. provide tolerance to pH extremes, pesticides, fertilizers,

and heavy metals (Ponmurugan and Shyamkumar, 2011). Endospore formation also confers bacterial stability during formulation and storage of products, thereby making it a valuable commercial bacterial inoculant (Kokalis-Burelle et al., 2006).

In order for a PGPR strain to be suitable and adaptable to field conditions, its compatibility with conventional fungicides is a pre-requisite. Strobilurin group of fungicides are commonly used in rice ShB management. Strobilurins an exceptional new group of fungicides capable of controlling diverse combination of phytopathogens, which was previously only possible using combination of one or more fungicides (Bartlett et al., 2001; Rodrigues et al., 2013). Azoxystrobin, a strobilurin group fungicide, is frequently used by rice growers worldwide. Azoxystrobin, along with related other products, are the world's premium fungicides. It is registered for crop use in over 70 countries by Syngenta (http://www.syngenta.com, accessed August 2012; Rodrigues et al., 2013). Compatibility of PGPR to azoxystrobin is necessary for devising biocontrol-based IDM strategies against rice ShB.

The current research work comprised two objectives. Objective 1 was to evaluate PGPR strains against three different pathogen isolates collected in the U.S. The second objective was to select a superior performing PGPR strain from objective 1 and evaluate its compatibility with commonly used fungicide in the U.S. Previous studies on *in vitro* screening of different PGPR strains and current study under greenhouse conditions proved that *B. subtilis* strain AP 301 was highly efficacious in reducing ShB pathogen and lesion spread. In the present study, the combined efficacy of AP 301 and azoxystrobin was evaluated at different rates of application to determine the optimum dose of bioagent and fungicide. The longterm goal is to reduce the fungicidal application and to minimize the environmental hazards and costs incurred in plant protection.

II. Materials and Methods

Fungi and bacteria used for experiment

A virulent *R. solani* inoculum isolated from field grown rice (cv. Cocodrie) infested with ShB disease was used for this research. The infectious sclerotia were collected by Dr. Shane Zhou (Assistant professor at Texas A&M University System, AgriLife Research & Extension Center, Beaumont, TX, USA). The pathogen was maintained on potato dextrose agar (PDA) prior to use.

All experimental treatments of PGPR strains were obtained from Dr. J. W. Kloepper laboratory culture collection (Professor at Department of Entomology and Plant Pathology, Auburn University, AL, USA). Before using PGPR for lab studies the strains were tested for purity as suggested by Kotamraju, 2010. PGPR strains were stored in tryptic soy broth (TSB) culture amended with 20% glycerol at -80^0 C. Bacterial cell suspensions were streaked on tryptic soy agar (TSA) plates and incubated for one day to check the purity. After 24 h of incubation, the bacterial cells or colonies were harvested from TSA plates in sterile distilled water and centrifuged for 5 min at 6000 rpm (Raj et al., 2012). Bacterial pellets were resuspended in sterile water and spectrophotometer was used to adjust PGPR treatments concentration or density to desired levels. The above purity check for PGPR treatments was performed during each assay and before they were applied as treatments.

Source of rice cultivar

Seeds of rice cultivar Cocodrie were obtained from Louisiana State University, research center located at Crowley, LS were used for this study. Rice seeds were stored under cool temperature conditions (4-5^0 C) before seeding. Cocodrie rice cultivation was successful in USA due to its early seed development and superior grain yielding ability. However, Cocodrie rice cultivar is susceptible to ShB disease causing huge economic yield losses. All rice experiments were conducted at plant science research center, Auburn, Alabama.

In vivo screening of PGPR under greenhouse conditions

Objective 1 was to evaluate the efficacy of different PGPR on rice ShB severity caused by three *R. solani* isolates under greenhouse conditions by adopting micro-chamber inoculation method. This objective was performed as suggested by Kotamraju, 2010. Seeds of rice were sown in 0.5 gallon plastic pots containing sterilized local soil (1 part) and peat-vermiculite potting mixture (2 parts). Once the soil is settled uniformly inside the pot, three seeds were sown per pot. After 15 days of rice seed emergence they were thinned back to one seedling per pot. Antagonistic PGPR strains prepared at a density of $1x10^9$ CFU/ml were sprayed onto seedlings at 3 leaf stage (30 days after sowing) until run off. After 24h of bacterial inoculation, immature sclerotia of *R. solani* were inoculated at the base of treated seedlings near soil line to produce ShB disease to evaluate the efficacy of different PGPR as a biocontrol agent. Seedlings inoculated with *R. solani* sclerotia and sprayed with water is inoculated/pathogen control. Seedlings sprayed with water and not inoculated with sclerotia is healthy control treatment. In total, there were 11 treatments, four replications per treatment, with one plant per replication. Treatment pots were arranged in a randomized complete block design (RCBD) and seedlings were allowed to grow for 45 days inside the nonchemical zone greenhouse chamber. Crop growing conditions were replicated inside the greenhouse by adjusting temperature, relative humidity, and photoperiod to 26 ± 2^0 C, 90±1%, and 16 h per day, respectively. Fifteen days after pathogen inoculation, ShB lesions' lengths and culm lengths were measured.

Assess the combined efficacy of PGPR and fungicide on rice ShB under controlled conditions

Objective 2 emphasis was to assess the combined efficacy of PGPR (strain AP 301) and azoxystrobin against rice ShB by adopting micro-chamber inoculation method. Experimental design used was a 5x7 factorial RCBD. Strain AP 301 was evaluated at concentrations of zero, 10^3, 10^6, 10^9 and 10^{11} CFU/ml (5 factors) in combination with azoxystrobin at zero, 396, 793,

1189, 1585, 1982, and at recommend rate (R) 2,378 ppm (7 levels). Azoxystrobin treatments were given in ascending order of 0, R/6, 2R/6, 3R/6, 4R/6, 5R/6 and recommended rate (R). The fungicide rate (R) for pot studies was calculated using recommended field applied rate of 0.17 kg ai/ha. Rice seeds were grown as suggested by Kotamraju, 2010. Rice seeds of cultivar Cocodrie were surface sterilized with 2% sodium hypochlorite for 5 min and washed with sterile distilled water twice. For seed treatment, surface sterilized seeds were soaked in different concentrations of AP 301 prepared by multiplying the strain on Tryptic Soy Agar (TSA) media in Petri dishes and adjusting to final concentrations. Later, seeds were soaked for 24 h separately. Seeds were later removed from the bacterial soaked solutions and air dried in a laminar flow hood for 30 minutes. Seeds were sown in 0.5 gallon plastic pots containing sterilized local soil (1 part) and peat-vermiculite mixture (2 parts). Once the soil is settled uniformly inside the pot, three seeds were sown per pot. After 15 days of rice seed emergence they were thinned back to one seedling per pot. At 3 leaf stage (30 days after sowing), plants were sprayed until run off with AP 301and azoxystrobin at different concentrations according to treatment combinations described previously. After 24 h of imposing the foliar spray with AP 301 and azoxystrobin, immature sclerotia of *R. solani* (Texas isolate) were inoculated at the base of treated seedlings near soil line to produce ShB disease to evaluate the efficacy of combined application of AP 301and azoxystrobin. Seedlings inoculated with *R. solani* sclerotia and with no seed treatment and foliar spray with AP 301 served as pathogen control. Seedlings that were produced by soaking seeds in water and later sprayed with water and not inoculated with sclerotia served as healthy control. Overall, there were 36 treatments, four replications per treatment, and single pot per repetition.

Pots were arranged in a randomized complete block design (RCBD) and the seedlings were allowed to grow for 45 days inside the chemical zone greenhouse chamber. The crop

growing conditions were replicated inside the greenhouse by adjusting the temperature, relative humidity, and photoperiod to 26 ± 2^0 C, 90±1%, and 16 h per day, respectively. Fifteen days after inoculation with pathogen, the ShB lesions' lengths and culm lengths were measured, and disease severity was rated.

Disease assessment

For both objectives, ShB disease was calculated by Relative lesion lengths (RLH) were calculated using a formula suggested by Sharma et al., 1990.

$$\text{RLH (\%)} = \frac{100(\text{Total lesion heights})}{\text{Total height of leaf}}$$

ShB disease severity grade scale (0 to 9) for two experimental objectives was given (Groth, 2005) as follows:

Disease severity was determined on a "0 to 9 scale" where **0**= Plants healthy, no symptoms; **1**= restricted dark brown oval lesions at waterline or infection points; **2**= few oval or coalesced lesions with broad borders on lower sheaths or at infection points, 5% or less of tissue affected; **3**= lesions on lower leaf sheaths or at infection points, lesions coalescing, less than 10% of tissue affected; **4**= lesions mainly restricted to sheaths on lower third of plant, lowest leaves, or other infection points, lesions discrete or coalescing with narrow red-brown border, 10 to 15% of leaf and sheath tissues affected ; **5**= lesions mainly restricted to sheaths and leaves of lower half of plants, lesions usually coalescing with large necrotic centers and narrow red-brown borders, 15 to 25% of tissues affected; **6**= lesions usually coalescing and affecting lower two-thirds of sheath area of plant, lesions extending to blades of lower leaves or lower leaves killed by injury to sheath, 25 to 40% of tissues affected; **7**= lesions usually coalescing and affecting lower three-fourths of sheath area of plant, lesions extending to leaf blades of lower two-thirds of plant, 40 to

60% of tissues affected; **8**= lesions reaching to flag leaf, lower sheaths with coalesced lesions covering most of tissue, lower and middle leaves dead or dying, 60 to 80% of tissues affected; and **9**= lesions reaching to flag leaf, lower leaves mostly dead, sheaths dried, culms brown, collapsing, most tillers lodged, over 80% of tissues affected.

Statistical analysis

Possible difference noted in the appearance of infection by *R. solani* isolates in response to PGPR treatments were analyzed using SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). Plant lesion heights and ShB disease severity were analyzed by restricted maximum likelihood (REML) approach using Proc-Mixed analysis. Significance of treatment averages were differentiated using Tukey's least significant difference test at probability level of 0.05. A simple regression analysis was performed for objective 2 to determine the relationship between treatments and measured disease parameters. Proc-Regression was used for regression analysis of experimental variables.

III. Results

In vivo screening of PGPR under greenhouse conditions

When compared to control, rice seedlings treated with bacterial strains had a significant difference ($P<0.05$) on *R. solani* (3 isolates) disease parameters related to lesion height and disease severity. Each pathogen isolate was tested separately against PGPR treatments. The total means of percent lesion height for the AK, MS, and TX isolates were 7.83 ± 0.37, 7.27 ± 0.29, and 8.13 ± 0.47%, respectively (Table 1). Among different PGPR strains screened against three isolates of pathogen, ShB lesion spread ranged from 1.62 ± 0.22 to 12.21 ± 0.59% (Table 1). Experiments with AK, MS, and TX isolates showed that three antagonists, AP 301, AP52, and AP 209, showed maximum reduction in lesion growth under greenhouse conditions (Figure 1). The highest reduction in disease lesion spread was obtained when seedlings inoculated with AK,

MS, and TX isolates were sprayed with *B. subtilis* AP 301 (Table 1). Compared to pathogen control, when sprayed with AP301 the lesion spread was reduced by 84.47, 83.82, and 86.54 % for AK, MS, and TX, respectively. Strains AP7, AP136, AP295, AP305, and AP294 showed more lesion spread than the average mean lesion spread.

The disease severity rating ranged from 1.25 ± 0.29 to 6.50 ± 0.28 (Table 2) for 3 pathogen isolates when plants were treated with different PGPR strains. The total mean disease severity for the AK, MS, and TX isolates were 3.80 ± 0.27, 4.05 ± 0.29, and 4.32 ± 0.31, respectively (Table 2). Strains AP 301, AP52, and AP 209 had lower disease severity grade than the treatment average means (Figure 2). When compared to pathogen control, the percent decrease in disease severity was observed for rice plants treated with AP301 against AK, MS, and TX isolates (79.20, 76.00, and 80.76%, respectively).

Assessing the combined efficacy of PGPR and fungicide on rice ShB under controlled conditions

Azoxystrobin fungicide at recommended rate (R) when used in conjunction with any of the concentrations of strain AP 301 (10^3, 10^6, 10^9 and 10^{11} CFU/ml) resulted in complete reduction of ShB lesions (0% severity by RLH) (Tables 3 and 4). In a similar way, strain AP 301 at a concentration of 10^{11} CFU/ml when applied in conjunction with any of the concentrations of azoxystrobin under study (R/6 through R) resulted in complete control of ShB lesions (0% ShB severity). Also, AP 301 at 10^{11} CFU/ml alone could completely inhibit ShB lesion development under greenhouse conditions. The combined application of AP 301 at any of the concentrations under study with azoxystrobin at recommended rate was significantly superior (0% ShB severity) over the application of azoxystrobin alone at recommended rate, R (4% severity by RLH). ShB lesion size was significantly less over control (pathogen control) in other treatments involving combinations of AP 301 @ 10^3 and azoxystrobin at different concentrations (Figure 3). However, complete control of ShB lesions was seen in the following treatments that do not

involve AP 301 strain at 10^{11} CFU/ml and azoxystrobin at recommended rate (R) as factors: AP 301 @ 10^{9} CFU/ml + azoxystrobin @ 1189 ppm; AP 301 @ 10^{9} CFU/ml + azoxystrobin @ 1585 ppm; AP 301 @ 10^{9} CFU/ml + azoxystrobin @ 1982 ppm; AP 301 @ 10^{9} CFU/ml + azoxystrobin @ recommended rate, R (2378 ppm).

IV. Discussion

PGPR are well known for their plant growth promotion properties on a wide range of hosts including rice. The current research under greenhouse conditions was focused on evaluating the capacity of selected PGPR strains to reduce disease incidence and their compatibility with a fungicide commonly used by growers in the U.S. Screening results from objective one proved the potential of pure culture PGPR to suppress disease under greenhouse conditions. Similar to our previous *in vitro* screening tests conducted under laboratory conditions, these results offered further evidence of PGPR's capacity to control rice ShB disease.

In the present study all *Bacillus* and one *Paenibacillus* species and subspecies tested showed reduced rice ShB lesion height and disease severity when compared to pathogen control treatments (Tables 1 and 2). Out of nine PGPR strains screened, three antagonists, *Bacillus subtilis* (AP 301), *Bacillus subtilis subsp. subtilis* (AP52), and *Bacillus mojavensis* (AP 209), showed consistent reduction of both relative lesion height and disease severity caused by all three isolates of *R. solani*. Highest reduction in ShB lesion spread (1.6% by RLH) with a concomitant ShB severity grade of 1.3 was obtained when seedlings were sprayed with *Bacillus subtilis* AP 301 against *R. solani* (TX isolate). When compared with the pathogen control, the highest percent reduction of lesion height was measured for strain AP 301 (86.54 %). PGPR also showed significant effect on disease severity when measured using 0-9 scale as described in materials and methods. Four PGPR strains, AP 301, AP 52, AP 305, and AP 209, showed 50% or more

reduced disease severity (caused by AK and MS isolates) when compared to pathogen control (Table 2).

One possible mechanism for ShB disease reduction in rice plants by PGPR is the activation of ISR. Rhizobacteria-mediated ISR may be defined as a plant's innate defensive capacity against plant pathogens that is elicited in response to specific environmental stimuli (van Loon, 2000). Upon pathogen infection in rice seedling, the mechanism of ISR is increased by high production of defense related enzymes and chemicals (Chithrashree et al., 2011). ISR in plants is often triggered by variety of defense compounds like β-1-3-glucanase, chitinase, PAL, PO, PPO, and rise in phenolic substances (Meena et al., 2000; Chithrashree et al., 2011). All the PGPR strains reduced lesion height when compared to pathogen control, indicating that ISR may have been triggered in rice seedlings.

Due to superior performance of strain AP 301, further studies for pesticide compatibility under greenhouse conditions were performed to determine the optimum dose of bioagent as well as fungicide in order to develop a biocontrol based IDM approach for rice ShB. Based on the results it can be inferred that *B. subtilis* strain AP 301 @ 10^9 CFU/ml, when used in combination with azoxystrobin @ 1189 ppm and above of the recommended rate, provided complete control of ShB lesion spread under greenhouse conditions (Tables 3 and 4). The combined application of AP 301 with azoxystrobin @ 1189 ppm of recommended rate is the optimum dose since the combination involved lower amounts of azoxystrobin. Hence, it can be concluded that combined application of *B. subtilis* AP 301 (@10^9 CFU/ml) and azoxystrobin (@1189 ppm or 3/6 of recommended rate) is an ideal dose of PGPR and fungicide.

In our greenhouse studies, the optimum combination of *B. subtilis* AP 301 (@10^9 CFU/ml) and azoxystrobin (@ 3/6 of recommended rate) was selected as it provided complete

inhibition of ShB lesions. Several other combinations of AP 301 and Azoxystrobin resulted in ShB lesion reduction significantly over pathogen control but did not provide complete inhibition. Complete inhibition is desired as the pathogen inoculum of *R. solani* under field conditions is manifold and the chances of disease outbreak are greater compared to greenhouse conditions. If the control of ShB disease under field conditions is regulated, the damage is notably less. Further studies are needed to evaluate these combined applications of *B. subtilis* AP 301 and azoxystrobin at various rates under field conditions to determine the efficacy and optimum dose for ShB reduction.

There was a significant regression between the ShB relative lesion height and treatments with a regression line $y = 15.14\text{-}0.46x$, coefficient differentiation $R^2 = 0.63$ (Figure 4); $y = 6.94\text{-}0.21x$, $R^2 = 0.66$ for disease severity and treatments (Figure 5). Regression analysis indicates that for unit increase in treatment concentrations applied, there was a decrease of 0.46 mm ShB relative lesion heights and 0.21 disease severity indexes.

The above findings demonstrate that some *Bacillus* strains are more effective than others in controlling rice ShB disease. Kloepper et al. (2004) reported that strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* significantly reduced disease incidence in tropical crops by triggering plant ISR. It is well known that *Bacillus* and closely related species are comprehensively studied PGPR due to their antagonistic abilities. *Bacillus* and *Paenibacillus* species are antagonistic to phytopathogens, which has been proven through both *in vitro* and *in vivo* research studies many times (Joshi and McSpadden Gardener, 2006; Chen et al., 2009; Arrebola et al., 2010; Kumar et al., 2011). *Bacillus* spp. effectiveness against ShB is due to its quick adaptability to the rhizosphere environment and its strong ability to compete with surrounding organisms.

Regardless of the significant success of PGPR in disease suppression under controlled conditions, they are seldom used in large rice growing field conditions compared to fungicides. However, due to the increase in strict implementation of anti-agricultural chemical usage laws by many countries, it is helpful to have an alternative disease management approach. Application of resistance inducing PGPR is effective, long-lasting, and harmless to the environment. These properties of PGPR could be a beneficial factor of other complementary disease protection programs commonly termed as IDM. Furthermore, for protection of rice crop against plant pathogens, PGPR have also proven to increase rice growth and yield. Future work should focus on a more comprehensive cost and benefit analysis to evaluate the economic feasibility of biological control of rice ShB in agricultural best management practices.

V. References

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VI. Appendix

Table 1. Effect of PGPR strains on percent relative lesion height spread by *Rhizoctonia solani* isolates collected from Arkansas, Mississippi, and Texas

***In vivo* percent (%) relative lesion height of *R. solani* isolates on rice leaf sheaths**					
Trt no.	**Strain**	**Identification**	**Arkansas (mm)**	**Mississippi (mm)**	**Texas (mm)**
T1	AP 301	*Bacillus subtilis*	1.99±0.38^{g}	2.06±0.33^{f}	1.62±0.22^{d}
T2	AP52	*Bacillus subtilis subsp. subtilis*	5.03±0.74^{f}	3.96±0.43^{e}	6.81±0.63^{c}
T3	AP7	*Bacillus safensis*	10.82±0.66^{ab}	9.96±0.53^{bc}	10.87±0.59^{ab}
T4	AP136	*Bacillus amyloliquefaciens*	10.36±0.28^{b}	10.21±0.45^{bc}	10.24±0.50^{ab}
T5	AP295	*Bacillus amyloliquefaciens*	9.93±0.59^{bc}	9.71±0.17^{c}	10.59±0.40^{ab}
T6	AP305	*Bacillus amyloliquefaciens subsp. plantarum*	7.91±0.14^{cd}	7.61±0.02^{d}	12.21±0.59^{a}
T7	AP188	*Bacillus amyloliquefaciens*	7.43±0.28^{de}	6.97±0.34^{d}	9.56±0.62^{b}
T8	AP294	*Paenibacillus peoriae*	11.12±0.36^{ab}	11.41±0.38^{ab}	11.34±0.59^{ab}
T9	AP209	*Bacillus mojavensis*	5.80±0.08^{ef}	5.32±0.32^{e}	4.16±0.62^{d}
T10	Pathogen control	-----	12.82±0.59^{a}	12.73±0.26^{a}	12.02±0.44^{a}
T11	Healthy control	-----	0.00	0.00	0.00
	Total Avg. Mean		7.83±0.37	7.27±0.29	8.13±0.47

% relative lesion spread (mm) measured at 15 days after pathogen inoculation on rice seedlings
Results are an average of 4 replicates ± standard error
Means or Means ± standard error followed by the similar alphabet are significantly not different according to Tukey's multiple range test at P = 0.05

Table 2. Effect of PGPR strains on ShB disease severity caused by *Rhizoctonia solani* isolates collected from Arkansas, Mississippi, and Texas

Disease severity caused by *R. solani* isolates on rice leaf sheaths under controlled conditions					
Trt no.	**Strain**	**Identification**	**Arkansas**	**Mississippi**	**Texas**
T1	AP 301	*Bacillus subtilis*	1.30±0.43^{e}	1.50±0.23^{f}	1.25±0.29^{c}
T2	AP52	*Bacillus subtilis subsp. subtilis*	2.5±0.40^{ef}	3.00±0.37^{ed}	3.25±0.29^{b}
T3	AP7	*Bacillus safensis*	5.00±0.16^{ab}	4.75±0.40^{bc}	5.25±0.28^{a}
T4	AP136	*Bacillus amyloliquefaciens*	6.25±0.24^{bc}	4.75±0.28^{bc}	5.75±0.36^{a}
T5	AP295	*Bacillus amyloliquefaciens*	6.25±0.24^{bc}	6.25±0.32^{bcd}	5.25±0.30^{a}
T6	AP305	*Bacillus amyloliquefaciens subsp. plantarum*	3.50±0.20^{cde}	3.50±0.23^{cde}	5.75±0.22^{a}
T7	AP188	*Bacillus amyloliquefaciens*	3.25±0.23^{ed}	6.25±0.32^{ed}	5.25±0.29^{a}
T8	AP294	*Paenibacillus peoriae*	4.00±0.27^{bcd}	5.50±0.23^{ab}	6.25±0.33^{a}
T9	AP209	*Bacillus mojavensis*	3.50±0.26^{cde}	2.75±0.20^{ef}	3.00±0.43^{b}
T10	Pathogen control	------	6.25±0.24^{a}	6.25±0.32^{a}	6.50±0.28^{a}
T11	Healthy control	------	0.00	0.00	0.00
	Total Avg. Mean	-----	3.80±0.27	4.05±0.29	4.32±0.31

Disease severity (0-9 scale) measured at 15 days after pathogen inoculation on rice seedlings
Results are an average of 4 replicates ± standard error
Means or Means ± standard error followed by the similar alphabet are significantly not different according to Tukey's multiple range test at P = 0.05

Table 3. Effect of strain AP 301and Azoxystrobin on ShB lesion height caused by *Rhizoctonia solani* isolate collected from Texas

Azoxystrobin (ppm)	**AP 301@ 0 CFU/ml**	**AP 301@ 10^3 CFU/ml**	**AP 301@ 10^6 CFU/ml**	**AP 301@ 10^9 CFU/ml**
0	14.42±0.33^{abc}	13.55±0.41^{bcde}	15.25±0.44^{a}	5.88±0.56^{h}
R/6	14.40±0.38^{abc}	14.02±0.39^{abcd}	14.29±0.72^{abcd}	6.46±0.40^{gh}
2R/6	13.68±0.46^{bcde}	13.33±0.50^{cde}	14.85±0.45^{ab}	5.84±0.36^{h}
3R/6	14.83±0.47^{ab}	12.27±0.43^{ef}	7.26±0.10^{gh}	0.00^{j}
4R/6	6.80±0.21^{gh}	12.89±0.66^{def}	7.34±0.10^{g}	0.00^{j}
5R/6	7.20±0.40^{gh}	11.75±0.67^{f}	7.20±0.22^{gh}	0.00^{j}
R	4.20±0.65^{i}	0.00^{j}	0.00^{j}	0.00^{j}

% Relative lesion spread (mm) measured at 15 days after pathogen inoculation on rice seedlings
Results are an average of 4 replicates
Means or Means ± standard error followed by the similar alphabet are significantly not different according to Tukey's multiple range test at P = 0.05
Treatments AP 301@10^{11} CFU/ml when applied in conjunction with azoxystrobin and healthy control showed no disease

Table 4. Effect of strain AP 301and Azoxystrobin on ShB disease severity caused by *Rhizoctonia solani* isolate collected from Texas

Azoxystrobin (ppm)	**AP 301@ 0 CFU/ml**	**AP 301@ 10^3 CFU/ml**	**AP 301@ 10^6 CFU/ml**	**AP 301@ 10^9 CFU/ml**
0	6.25±0.21^{abc}	6.25±0.21^{abc}	6.75±0.22^{a}	3.25±0.21^{f}
R/6	6.25±0.29^{abc}	5.50±0.34^{de}	6.50±0.28^{ab}	3.50±0.31^{f}
2R/6	6.75±0.30^{a}	6.00±0.39^{bcd}	6.50±0.31^{ab}	3.25±0.25^{f}
3R/6	6.25±0.29^{abc}	5.25±0.28^{e}	3.75±0.24^{f}	0.00^{g}
4R/6	3.25±0.25^{f}	5.50±0.27^{de}	3.25±0.29^{f}	0.00^{g}
5R/6	3.50±0.28^{f}	5.75±0.47^{cde}	3.25±0.21^{f}	0.00^{g}
R	3.75±0.24^{f}	0.00^{g}	0.00^{g}	0.00^{g}

Disease severity (0-9 scale) measured at 15 days after pathogen inoculation on rice seedlings
Results are an average of 4 replicates
Means or Means ± standard error followed by the similar alphabet are significantly not different according to Tukey's multiple range test at P = 0.05
Treatments AP 301@10^{11} CFU/ml when applied in conjunction with azoxystrobin and healthy control showed no disease

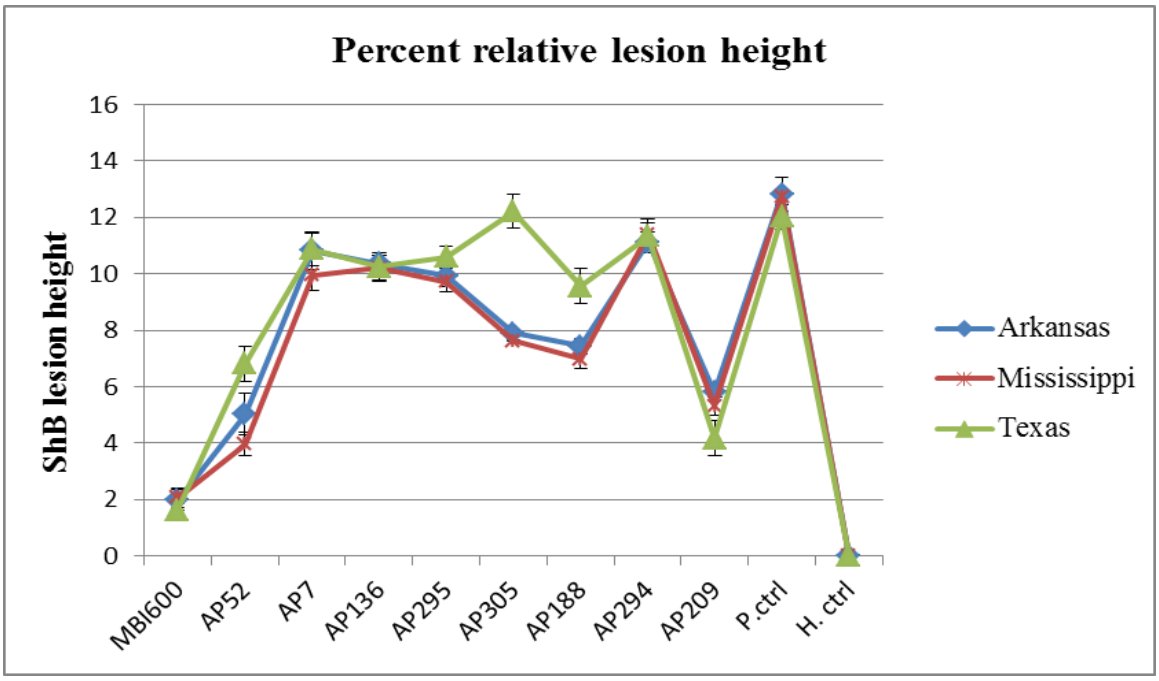

Figure 1. Influence of PGPR treatments on percent relative lesion spread (mm) on rice by *Rhizoctonia solani* isolates collected from Arkansas, Mississippi, and Texas. The error bars shown represent ± S.E.

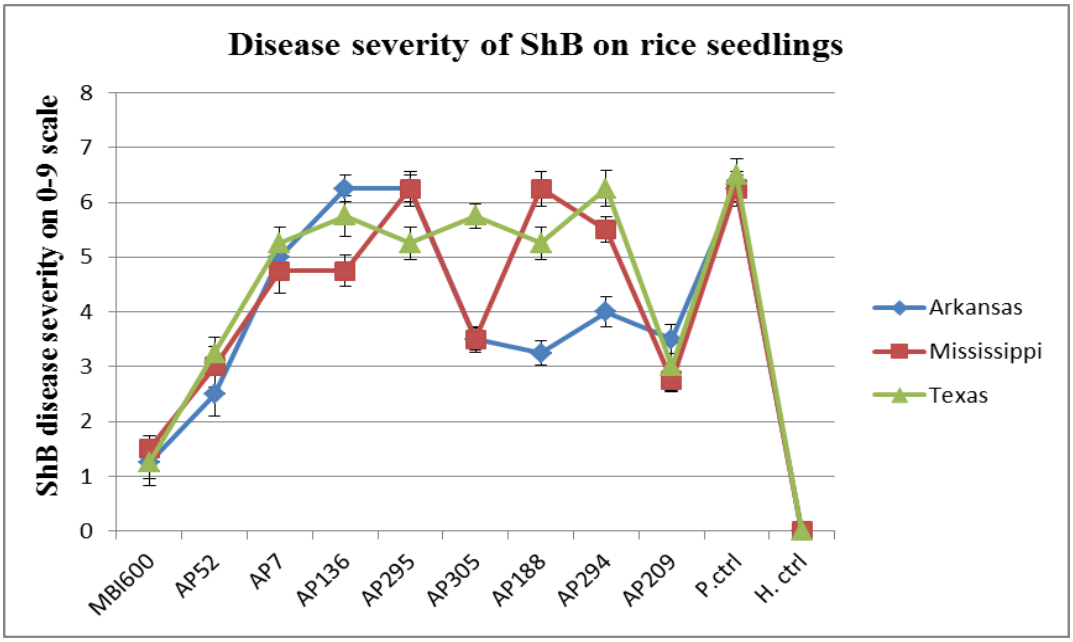

Figure 2. Influence of PGPR treatments on ShB disease severity on rice seedlings caused by *Rhizoctonia solani* isolates collected from Arkansas, Mississippi, and Texas. The error bars are shown represent ± S.E.

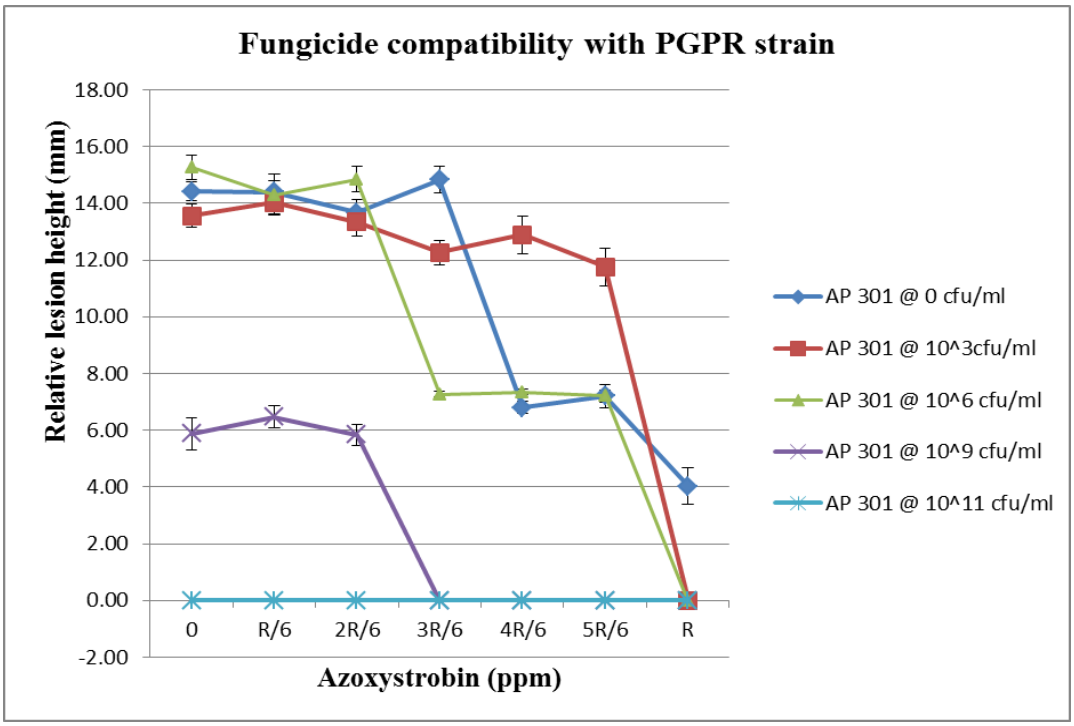

Figure 3. Effect of PGPR and fungicide treatments on reduction of ShB relative lesion height on rice caused by *Rhizoctonia solani* isolate collected from Texas. The error bars are shown represent ± S.E.

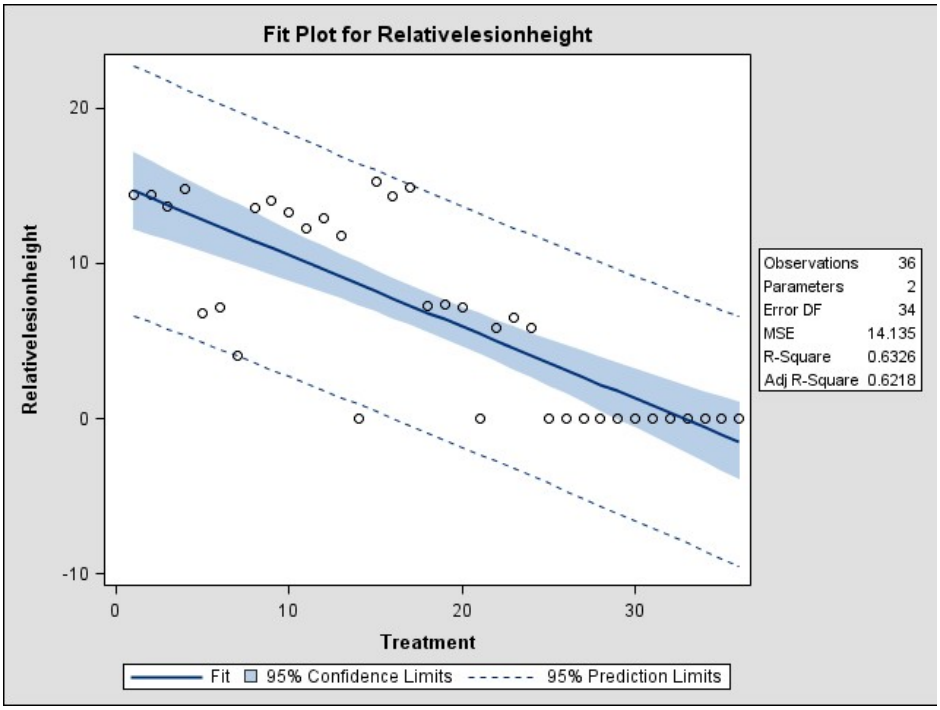

Figure 4. Regression analysis fit plot for relative lesion height against 36 treatments. It is estimated that median relative lesion height decreases with increase in each treatment concentration.

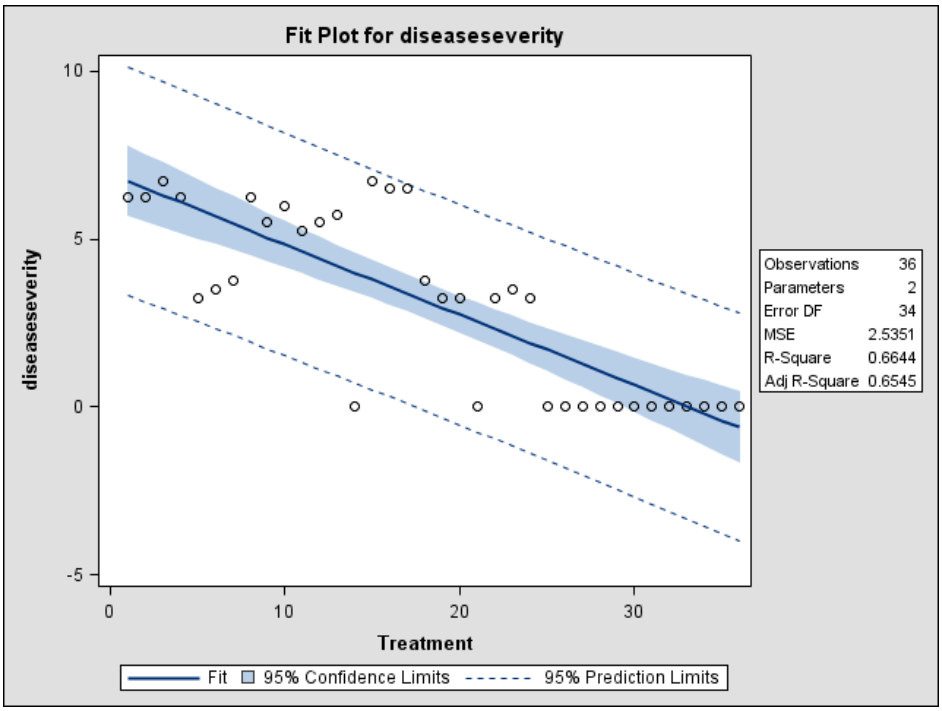

Figure 5. Regression analysis fit plot for disease severity against 36 treatments. It is estimated that median disease severity decreases with increase in each treatment concentration.

Chapter IV. Compatibility of fertilizers and plant growth-promoting rhizobacteria (PGPR) applied for inhibition of rice sheath blight under greenhouse conditions

Abstract

Sheath blight (ShB) is a major soilborne disease causing major economic losses to rice cultivation. Improper use of fertilizers by growers aids in pathogen dissemination and aggravation. Most of the prevalent disease control methods are focused against the pathogen directly and have been moderately successful. There is a need to develop an alternative control method that not only reduces ShB disease but also minimizes the use of excess fertilizers. In the experiments (summer and fall), a novel ShB management strategy was evaluated by optimizing concentrations of plant growth-promoting rhizobacteria (PGPR) and rates of fertilizers. PGPR at $1x10^9$ CFU/ml density was blended with different rates of nitrogen (N) and potassium (K) to evaluate their effect on ShB disease spread and their subsequent effect on rice yields. Pot culture experiments were treated with high, low, and recommended rates of fertilizers to determine the optimum dose of PGPR and fertilizer under controlled conditions. PGPR combined with N fertilizer applied at half the recommended rate resulted in lowest disease lesion spread up to 2.83±0.15 mm and 2.33±0.16 mm for summer and fall experiments, respectively. For summer experiment, the treatment of PGPR combined with N applied at half recommended rate produced higher yields (23.12±0.33 g) than that of treatments applied using higher N rates. However, application of treatments consisting of PGPR and high rates of N fertilizer has produced lower yields ranging from 20.55±0.30 and 17.94±0.89 g. The experiment was repeated again in fall 2012, and our treatments showed a similar trend of disease and grain weight results. These results indicate that using PGPR in conjunction with fertilizers will minimize ShB disease incidence and lower crop fertilizer inputs, thereby providing a feasible and sustainable rice production.

I. Introduction

Rice sheath blight (ShB) is considered the most damaging of the three major rice diseases occurring today (Li et al., 2012). ShB is caused by *Rhizoctonia solani* Kuhn (Teleomorph: *Thanatephorus cucumeris* (Frank) Donk) and is a destructive disease worldwide causing significant yield loss and quality degradation (Lee and Rush, 1983; Nagarajkumar et al., 2004).The pathogen also infects many other plant species, including barley, lettuce, tomato, sorghum, and maize (Zhang et al., 2009). A significant amount of achievable rice production is safeguarded from *R. solani* by using protection strategies. In their absence, rice ShB disease causes 10-30% yield loss (Xie et al., 2008) and may reach up to 50% during prevalent years (Meng et al., 2001). Planting ShB susceptible rice varieties in the U.S. resulted in yield losses of about 50% (Prasad and Eizenga, 2008; Zhang et al., 2009). In Arkansas, ShB was found in 50-66% of rice fields, causing 5-15% yield losses in 2001 (Annou et al., 2005; Tan et al., 2007). The above findings indicate that ShB is a serious rice disease worldwide.

Rhizoctonia solani is soilborne with limited movement due to lack of spores and survives in unfavorable conditions by forming sclerotia or dormant mycelia (Sumner, 1996; Anees et al., 2010). Sclerotia in soil can survive for 2 years and are spread during field preparation and flooding the field for irrigation (Webster and Gunnell, 1992; Brooks, 2007). During permanent flooding, the sclerotia may float and move within the field or to bordering fields through continuous flood irrigation. Sclerotia or hyphae attach to the plant, infecting and causing ShB disease, and the pathogen spreads under conditions favorable to disease development. Soilborne pathogens normally are dormant and immobile in the field, so the host plant typically grows towards the stationary pathogen (Gilligan, 1983; Anees et al., 2010). Upon contact with rice plants, mature sclerotia cause ShB infection. Initial symptoms occur on leaf sheaths near the water line as water-soaked lesions. Secondary infections are caused by hyphae growing upward

towards uninfected plant parts, producing additional lesions and sclerotia on leaf sheaths to complete the disease cycle (Webster and Gunnell, 1992; Brooks, 2007).

The use of high rates of N fertilizer, double cropping, and high plant densities enhance ShB severity in most of the world's rice growing areas (Lee and Rush, 1983; Nagarajkumar et al., 2004). In addition, plant morphological traits of rice cultivars are known to enhance ShB severity. The planting of semi-dwarf cultivars with more tillers caused serious rice ShB in southern China (Liao et al., 1997; Zuo et al., 2009). Studies conducted on relationship between plant morphological characters and their relation with ShB development confirmed that plant height alters microclimate and light transmission inside the dense canopy, thereby facilitating disease development (Han et al., 2003; Tang et al., 2007).

Growers commonly apply rates of N that are higher than those recommended for rice. This results in lush green vegetative growth conducive to pathogen spread. Application of excess N is not only hazardous to the soil and water environments but also increases rice ShB severity. Rice produces tillers when high N doses are applied, which increases its susceptibility to pathogens and insects. Rice ShB disease intensity and incidence increase with increased rates of N fertilizer application (Groth and Bollich, 2000). High N rates also facilitate the ShB disease development and spread by increasing tiller density and moisture retention inside the rice canopy (Savary et al., 1995; Tang et al., 2007). Therefore, careful N fertilizer management should be adopted for rice crop, which would increase nitrogen use efficiency during the growing season and diminish ShB disease concerns.

Potassium (K) is another major essential element which has a role in ShB disease because it is involved in resistance mechanisms of the host plant (Cakmak, 2005; Li et al., 2010). However, the mechanism by which K stimulates resistance towards a pathogen is not completely

understood (Amtmann et al., 2008). Dordas (2008) reported that K-deficient plants are highly susceptible to disease due to the alteration of metabolic functions of K in plant physiology. The most commonly accepted explanation for the mechanism by which K enhances plant resistance to pathogens is from the mechanical resistance point of view. This involves development of a thicker cell wall of epidermal cells in the presence of K (Mengel et al., 2001; Li et al., 2010). Experiments conducted on maize stock rot disease showed that K-applied plant cells have abundant golgi bodies, which could produce a high amount of secretions to degrade the mycelia (Li et al., 2010).

Studies on use of ecofriendly plant beneficial soil microorganisms have been gaining importance for ShB management. Among these organisms, the most widely studied group by plant pathologists is plant growth promoting rhizobacteria (PGPR). Besides their plant growth promoting properties they are well known phytopathogen antagonists. Several phytopathogenic (fungal, bacterial, and viral) diseases under greenhouse and fields conditions were effectively managed using PGPR mediated induced systemic resistance (ISR) (Kloepper et al., 2004; Zhang et al., 2010). *In vivo*, ISR is activated by pathogen attack and is followed by systemic production of several defense compounds (Dean and Kuc, 1985; Senthilraja et al., 2013). These compounds suppress pathogen invasion by mechanisms like cytolysis, leakage of potassium ions, and inhibition of mycelial growth and protein biosynthesis (Quan et al., 2010; Laslo et al., 2012).

Beneficial bacterial populations belonging to various genera thrive in rice ecosystems. Genus *Bacillus* is considered to be one of the most diverse and comprehensively studied PGPR groups (Garbeva et al., 2003; Beneduzi et al., 2008). Several *Bacillus* and *Paenibacillus* were used commercially for developing common plant growth promoters and biological fungicides, insecticides, and nematicides (Beneduzi et al., 2008). In addition, some *Bacillu*s sp. have shown

increased plant growth and yield (Pal and Jalali, 1998; Ponmurugan and Shyamkumar, 2011). Application of bacilli strains along with other disease control methods in an integrated practice in rice field may be a long-term, sustainable alternative to ShB control.

The current research work comprised two experiments conducted under greenhouse conditions. The experiment conducted during summer of 2012 is designated as summer experiment, and was repeated in fall 2012 and noted as fall experiment. Previous studies on PGPR-fungicide compatibility under greenhouse conditions proved that *Bacillus subtilis subsp. subtilis* strain AP 52 was highly efficacious in reducing ShB pathogen and lesion spread. In the present study, the efficacy of AP 52 in combination with different rates of fertilizer N and K was evaluated to determine the optimum dose of bioagent and fertilizer. The longterm goal is to reduce the chemical application and to minimize the environmental hazards and costs incurred in plant protection.

II. Materials and Methods

Fungi and bacteria used for experiment

A virulent *R. solani* inoculum isolated from field grown rice (cv. Cocodrie) infested with ShB disease was used for this research. The infectious sclerotia were collected by Dr. Shane Zhou (Assistant Professor at Texas A&M University System, AgriLife Research & Extension Center, Beaumont, TX, USA). The pathogen was maintained on potato dextrose agar (PDA) prior to use.

The treatment strain of PGPR was obtained from Dr. J. W. Kloepper's laboratory culture collection (Professor at Department of Entomology and Plant Pathology, Auburn University, AL, USA). For PGPR treatment, *Bacillus subtilis subsp. subtilis* (AP 52) was used throughout the experiment. Before using PGPR for research conducted within, bacterial strains were tested for purity as recommended by Kotamraju (2010). PGPR strains were stored in tryptic soy broth

(TSB) culture amended with 20% glycerol at -80^0 C until further analysis. Bacterial cell suspensions were streaked on tryptic soy agar (TSA) plates and incubated for one day to check the purity. After 24 h of incubation, the bacterial cells or colonies were harvested from TSA plates in sterile distilled water and centrifuged for 5 min at 6000 rpm (Niranjan Raj et al., 2012). Bacterial pellets were resuspended in sterile water, and a Turner spectrophotometer was used to adjust PGPR treatments' concentration to 1 x 10^9 CFU/ml. The above purity check was performed for all PGPR treatments used.

Source of rice cultivar

Seeds of rice cultivar Cocodrie were obtained from Louisiana State University, research center located at Crowley, LA, were used for this study. Rice seeds were stored under cool temperature conditions (4-5^0 C) before seeding. Cocodrie cultivar was successful in the USA due to its early seed development and superior grain yielding ability. However, Cocodrie rice variety is susceptible to ShB disease, which causes large yield losses. All rice experiments were conducted at Plant Science Research Center, Auburn, Alabama.

Fertilizer types

In this study, two fertilizers sources were used to supply rice with N-P-K nutrients. Nitrogen was derived from traditional fertilizer urea (32-00-00) manufactured by professional choice premium fertilizer company. Phosphorus and potassium nutrients were available to rice plants through application of mono potassium phosphate fertilizer (00-52-34) contrived from Haifa company. Fertilizer N was applied in split applications to rice, with 60% applied during seeding and 40% at internode elongation. Fertilizers P and K were used as single application before rice seeding.

In vivo rice seed assay

Greenhouse experiments were conducted in summer and fall 2012 at Plant Science Research Center, Auburn University, AL. Rice seedlings are produced in 0.5 gallon pots containing

autoclaved field soil. Soil type used in this experiment can be grouped as loamy to clay with CEC of 4.6-9.0 $cmol_c kg^{-1}$ and alkaline nature with pH 7.6 (Figure 1). Before pot filling, a soil sample was sent to Auburn University soil diagnostic center to assess the levels of macronutrients N, P, and K (Figure 1). Prior to transplanting, rice seeds of CV. Cocodrie were grown in plastic seed trays for 1 week. Once the soil was settled uniformly inside the pot, one healthy rice seedling was transplanted into the wet soil.

Fertilizers N, P, and K were applied at recommended rate of 2.2, 0.65, and 0.15 g/pot, respectively. N, P, and K recommended rates for rice pot culture was calculated using soil test report (Figure 1). Fertilizers were applied to loamy soil in the ratio of 240:80:120 Kg/ha (Singh et al., 2005 and Bahuguna et al., 2012). Fertilizers N and K were applied at different rates for different treatment combinations (Tables 1 and 2). Phosphorus fertilizer was applied uniformly throughout the experiment. Azoxystrobin fungicide at recommended rate of 2,378 ppm was sprayed until run off at 30 days after sowing as per the treatment combination (Kotamraju 2010). Overall, there were 7 treatments, four replications per each treatment, and one pot per repetition (Tables 1 and 2). Pots were arranged in a randomized complete block design (RCBD) and grown for 106 days inside the chemical zone greenhouse chamber.

Antagonistic PGPR strain at a density of $1x10^9$ CFU/ml was sprayed onto rice seedlings at 3rd leaf stage (30 days after sowing) until run off. After 24h of bacterial inoculation, mature sclerotium of *R. solani* (TX isolate) was inoculated at the base of rice seedlings to produce ShB disease. For inoculation, sheaths of second expanded leaves of 30-day-old plants were selected (Kotam raju, 2010). The leaf sheaths were unwrapped carefully and sclerotia were inoculated individually by placing one sclerotium per plant. The inoculated portion was sealed with cellophane tape and watered immediately. Fifteen days after inoculation with pathogen, the ShB

lesions' lengths and culm lengths were measured by a formula of relative lesion height methods (RLH) as suggested by Sharma et al. (1990):

$$\text{RLH (\%)} = \frac{100(\text{Total lesion heights})}{\text{Total height of leaf}}$$

The plants were harvested at 106 days after sowing. Observations were made for number of ear or panicle bearing tillers, number of grains per panicle, and total grain weight per plant.

Statistical analysis

Possible differences of *R. solani* responses to PGPR treatments were analyzed using SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). Plant lesion heights and ShB disease severity were analyzed by restricted maximum likelihood (REML) approach using Proc-Mixed analysis. The significance of treatment averages were differentiated using Tukey's least significant difference test at probability level of 0.05. Correlation and simple regression analyses were performed to determine the relationship between treatments and measured disease and plant yield parameters. Proc-Regression was used for regression analysis of experimental variables.

III. Results

Summer experiment, 2012

When compared to pathogen control, rice seedlings treated with bacterial strains had significant difference ($P<0.05$) on ShB lesion appearance caused by *R. solani*. Among different treatments tested against pathogen, ShB lesion spread ranged from 0.53 ± 0.10 to 14.25 ± 0.62% (Table 1). The lowest disease spread (0.53 ± 0.10%) was observed with treatment combination of PGPR-azoxystrobin-fertilizer. The total means of ear bearing panicles, total number of grains, and grain weight per plant for different treatments were 11.64 ± 0.60, 108.11 ± 1.60, and 20.82 ± 0.48, respectively (Table 1). The number of ear bearing rice tillers ranged from 8.25 ± 0.56 to 15.50 ±

0.43. Treatment AP 52+1.5N+K@R had the highest number of ear bearing tillers, up to 15.50 ± 0.43. The highest grain number per panicle (118.50 ± 1.88) and total grain weights (24.84± 0.39) were recorded for healthy control treatment.

There was a positive correlation observed between the independent variable (treatment) and experimental measurable variables. The correlation R^2 values for measurable variables RLH, ear bearing panicles, total number of grains, and grain weight per plant were 0.06, 0.03, 0.2, and 0.0005, respectively (Figures 10a, 10b, 10c, and 10d). There was a significant regression between the number of grains and the treatments with a regression line y= 100.17-1.98x, coefficient differentiation R^2= 0.2 (Figure 10c).

Fall experiment, 2012

PGPR treated rice seedlings had a significant effect (P or α<0.05) on ShB lesions caused by *R. solani*, when compared with pathogen control treatment. Among different treatments tested against pathogen, ShB lesion spread averages ranged from 0.15 ± 0.22 to 16.70 ± 0.67% (Table 2). Similar to summer experiment, the lowest disease spread (0.15 ± 0.22%) was observed with treatment combination of PGPR-azoxystrobin-fertilizer. Total means of ear bearing panicles, total number of grains, and grain weight per plant for different treatments were 11.29 ± 0.54, 111.39 ± 2.75, and 21.23 ± 0.49, respectively (Table 2). In total, the mean number of ear bearing rice tillers ranged from 8.50 ± 0.39 to 14.25 ± 0.73. The highest number of grains per panicle (118.50 ± 2.94) was observed when rice seedlings were treated with AP 52+1.5N+K@R. Treatment seven, applied with only recommended rate of fertilizer and no pathogen, had the highest total grain weight (g) per rice plant (25.71± 0.47).

A positive Pearson correlation coefficient was observed between the independent variable (treatment) and experimental measurable variables. The correlation R^2 values for measurable variables RLH, ear bearing panicles, total number of grains, and grain weight per plant were

0.03, 0.12, 0.003, and 0.02, respectively (Figures 11a, 11b, 11c, and 11d). There was no difference for regression model plotted between treatments and dependent variables at P level 0.05. However, there was a significant regression (at P level 0.1) between the number of ear bearing tillers observed and the treatments with a regression line $y = 10.32\text{-}0.31x$, P value (α) = 0.06.

IV. Discussion

Complete and successful management of rice ShB epidemics has not been achieved. This may be due to the multiple interactive crop factors involved in enhancing rapid spread of pathogen. For example, agronomic factors like use of high rates of N fertilizer, double cropping, and high crop density have been shown to increase ShB severity (Lee and Rush, 1983; Nagarajkumar et al., 2004). The current research objective was to minimize the role of fertilizers in rice ShB enhancement by using PGPR applications.

In the present study (summer and fall), PGPR used in conjunction with lower rates of N fertilizer showed significant disease reduction when compared with PGPR applied in conjunction with higher rates of fertilizer (Figures 2 and 6). PGPR combined with N fertilizer applied at half the recommended rate resulted in sheath lesion spread up to 2.83±0.15 mm (Table 1) and 2.33±0.16 mm (Table 2) for summer and fall experiments, respectively. In both experiments, the lowest disease spread was observed when PGPR-fertilizer-chemical combination is applied. Visible lesion spreads were measured at 0.53±0.10 (Table 1) and 0.15±0.22 mm (Table 2).

Of many possible mechanisms reported, PGPR-triggered ISR is the most studied in regard to reduction of plant diseases. Rhizobacteria-mediated ISR may be defined as a plant's innate defensive capacity against plant pathogens that is elicited in response to specific environmental stimuli (van Loon, 2000). Upon pathogen infection in rice seedling, the mechanism of ISR is

increased by high production of defense related enzymes and chemicals (Chithrashree et al., 2011). ISR in plants is often triggered by a variety of defense compounds like β-1-3-glucanase, chitinase, PAL, PO, PPO, and rise in phenolic substances (Meena et al., 2000; Chithrashree et al., 2011). All PGPR treatments combinations reduced lesion height when compared to pathogen control, indicating that ISR may have been triggered in rice seedlings.

Beneduzi et al. (2008) reported N is the most important fertilizer input applied during rice production as it is known to have critical effect on yield. However, application of N in excess of recommended rates leads to increased production of rice tillers, which results in formation of a canopy microclimate which is conducive to rapid ShB development (Kotamraju, 2010). An alternative is to use PGPR in combination with reduced N fertilizer rates as a supplement to plant nutrients. It is very important to study the use of PGPR as they fix nitrogen biologically and produce secretions that enhance rice growth (Verma et al., 2001; Beneduzi et al., 2008). In order to observe the effect of using reduced rates of N fertilizer alongside PGPR, rice yield parameters were estimated during harvest for two successive production seasons.

For the summer experiment, rice plants were harvested at 106 days, and yield responses were recorded for various rates of fertilizer treatments applied (Figures 2, 3, 4, and 5). Treatment PGPR with N applied at half recommended rate produced higher yields (23.12±0.33 g) than that of treatments applied using higher N rates (Table 1). However, there were lower total number of ear bearing tillers (8.25±0.56) and lower grain number per panicle (97.50±1.31) with reduced fertilizer treatment. The lower number of tillers can be correlated to the above finding of lower disease incidence with use of reduced rates of N fertilizer. However, application of treatments consisting of PGPR and high rates of N fertilizer produced lower yields ranging from 20.55±0.30 to 17.94±0.89 g (Table 1). Our findings were similar to previous research. It was reported that

excessive N inputs in rice resulted in reduced grain yield due to lodging and greater pest and pathogen incidence (Peng et al., 2010; Li et al., 2012). Yield reduction of up to 20 to 42% was reported when rice was inoculated with treatment of high N rate (Cu et al., 1996; Li et al., 2012).

The experiment was repeated again in fall 2012, and our treatments showed similar trends of disease and grain weight results (Figures, 6, 7, 8, and 9). Application of treatments having PGPR and N in ascending rates has disease lesion spread of 2.33±0.16, 3.34±0.32, and 6.91±0.38 mm (Table 2). Similarly rice grain yields showed corresponding decline with increasing rates of N fertilizer of 22.78±0.45, 20.70±0.75, and 18.20±0.70 g (Table 2). PGPR applications with varied rates of N fertilizer reduced or postponed disease development, thereby minimizing grain yield losses (Di-qin et al., 2012). However, the number of grains counted per panicle was higher with increased application rates of N fertilizer (Tables 1 and 2). The possible explanation for higher grain number and lower grain weight is that ShB infection resulted in unfilled or partially filled grains (Kotamraju, 2010).

In both experiments, we found that fertilizer K has significant influence on disease spread when applied at varied rates (Tables 1 and 2). This supports the findings by Dordas (2008) in which K-deficient plants were highly susceptible to disease due to the alteration of metabolic functions of K in plant physiology. However, when K was applied at recommended and half the recommended rate, there was no significant difference observed for grain weight per panicle (Table 2). Grain weights per panicle were 20.70±0.75 at the recommended rate and 20.75±0.44 g at half the recommended rate (Table 2). This observation suggests that K had minimum or no role in influencing rice yields under controlled conditions. Except for healthy control, the high disease reductions and greater grain yields were obtained with treatment combination (PGPR-fertilizer-azoxystrobin), when applied at recommended rate (Tables 1 and 2). Enhanced

performance may be explained in terms of application of dual ShB disease control products (PGPR and fungicide).

In conclusion, PGPR, when applied with lower rates of N fertilizer, had significant effect on ShB disease spread in comparison to treatments with higher rates. PGPR in conjunction with different N fertilizer rates had a significant influence on rice grain yields. Regardless of the significant success of PGPR in disease suppression and improved growth and yields under controlled conditions, PGPR are seldom used in large rice growing field conditions compared to fungicides and fertilizers. However, due to the increase in strict implementation of chemical usage laws by many countries in the area of agriculture, it is helpful to have an alternative disease management approach. Application of resistance inducing PGPR is effective, long-lasting, and harmless to the environment. Future work should focus on a more comprehensive cost and benefit analysis to evaluate the economic feasibility of biological control of rice ShB in agricultural best management practices.

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VI. Appendix

Table 1. Effect of treatments on percent relative lesion height, ear bearing tillers, grain weight, and total number of grains for rice grown under controlled conditions during summer 2012

Treatments	RLH	Ear bearing tillers/Panicle no.	Grain no./Panicle	Grain wt./Plant (g)
AP 52+0.5N+K@R	2.83±0.15^{d}	8.25±0.56^{c}	97.50±1.31^{c}	23.12±0.33^{a}
AP 52+ NK@R	4.49±0.38^{c}	12.00±0.68^{b}	106.25±1.90^{abc}	20.55±0.30^{b}
AP 52+1.5N+K@R	6.38±0.39^{b}	15.50±0.43^{a}	115.00±1.61^{ab}	17.94±0.89^{cd}
AP 52+0.5K+N@R	4.26±0.13c^{d}	11.00±0.76^{b}	103.50±1.66^{b}	20.22±0.45^{bc}
AP 52+NK@R+Azoxy	0.53±0.10^{e}	11.25±0.68^{b}	112.00±1.27^{abc}	23.34±0.32^{a}
Path ctrl+Fert NK@R	14.25±0.62^{a}	11.50±0.67^{b}	104.00±1.55^{abc}	15.76±0.70^{d}
Hlth ctrl+Fert NK@R	---	12.00±0.43^{b}	118.50±1.88^{a}	24.84±0.39^{a}
Total Avg. Mean	4.68±0.25	11.64±0.60	108.11±1.60	20.82±0.48

Alphabet 'R' in treatments' column indicates recommended rate
% Relative lesion spread (mm) measured at 15 days after pathogen inoculation on rice seedlings
Ear bearing tiller number, grain number, and grain weight were recorded at the time of harvest (@ 106 days)
Results are an average of 4 replicates
Means or Means ± standard error followed by the same letter are not significantly different according to Tukey's multiple range test at P = 0.05

Table 2. Effect of treatments on percent relative lesion height, ear bearing tillers, grain weight, and total number of grains for rice grown under controlled conditions during fall 2012

Treatments	RLH	Ear bearing tillers/Panicle no.	Grain no./Panicle	Grain wt./Plant (g)
AP 52+0.5N+K@R	2.33±0.16^{d}	8.50±0.39^{c}	107.75±3.15^{ab}	22.78±0.45^{bc}
AP 52+ NK@R	3.34±0.32^{cd}	11.75±0.60^{b}	114.25±4.03^{ab}	20.70±0.75^{c}
AP 52+1.5N+K@R	6.91±0.38^{b}	14.25±0.73^{a}	118.50±2.94^{a}	18.20±0.70^{d}
AP 52+0.5K+N@R	3.88±0.17^{c}	11.25±0.27^{b}	102.00±3.71^{b}	20.75±0.44^{c}
AP 52+NK@R+Azoxy	0.15±0.22^{e}	10.75±0.31^{b}	115.00±1.81^{ab}	24.41±0.31^{ab}
Path ctrl+Fert NK@R	16.70±0.67^{a}	12.25±0.73^{b}	105.75±0.94^{ab}	16.06±0.32^{d}
Hlth ctrl+Fert NK@R	-----	12.25±0.73^{b}	116.50±2.70^{a}	25.71±0.47^{a}
Total Avg. Mean	4.75±0.27	11.29±0.54	111.39±2.75	21.23±0.49

Alphabet 'R' in treatments' column indicates recommended rate
% Relative lesion spread (mm) measured at 15 days after pathogen inoculation on rice seedlings
Ear bearing tiller number, grain number, and grain weight were recorded at the time of harvest (@ 106 days)
Results are an average of 4 replicates
Means or Means ± standard error followed by the same letter are not significantly different according to Tukey's multiple range test at P = 0.05

Report on Soil Test

Auburn University Soil Testing Laboratory

Auburn University, AL 36849-5411

Shashi KR Yellareddygari
209 Life Sciences Bldg
Auburn University, AL 36489

County:Lee
District:2
Test Date:04/09/12

				SOIL TEST RESULTS				RECOMMENDATIONS			
LAB No.	Sample Designation	Crop	Soil Group*	pH**	Phosphorus P***	Potassium K***	Magnesium Mg***	Calcium Ca***	LIME-STONE	N	P_2O_5	K_2O
					Pounds/Acre				Tons/Acre	Pounds/Acre		
19445	XYZ	Research	2	7.6	10	95	498	1431	0.0	- -	- -	- -

The number of samples processed in this report is: 1

For further information call your county agent: (334) 749-3353

* 1. Sandy soil (CEC < 4.6 $cmol_c kg^{-1}$)
* 2. Loams and Light clays (CEC = 4.6-9.0 $cmol_c kg^{-1}$)
* 3. Clays and soils high in organic matter (CEC > 9.0 $cmol_c kg^{-1}$)
* 4. Clays of the Blackbelt (CEC > 9.0 $cmol_c kg^{-1}$)

** 7.4 or higher - Alkaline - - - - - - - - - - - - - - 6.6-7.3 - Neutral - - - - - - - - - - - - - 6.5 or lower - Acid - - - - - - - - - - - - -5.5 or lower - Strong Acid

*** Extractable nutrients in pounds per acre

If soil group = 1, 2 or 3, Method of Analysis = Mehlich-1. If soil group = 4, Method of Analysis = Miss/Lancaster.

Approved by: Print Date: April 9, 2012 Page 1 of 1

Figure 1. Soil test report on experimental pot soil used to grow rice seedlings. Soil diagnosis was performed by Auburn University Soil Diagnostic Lab, AL.

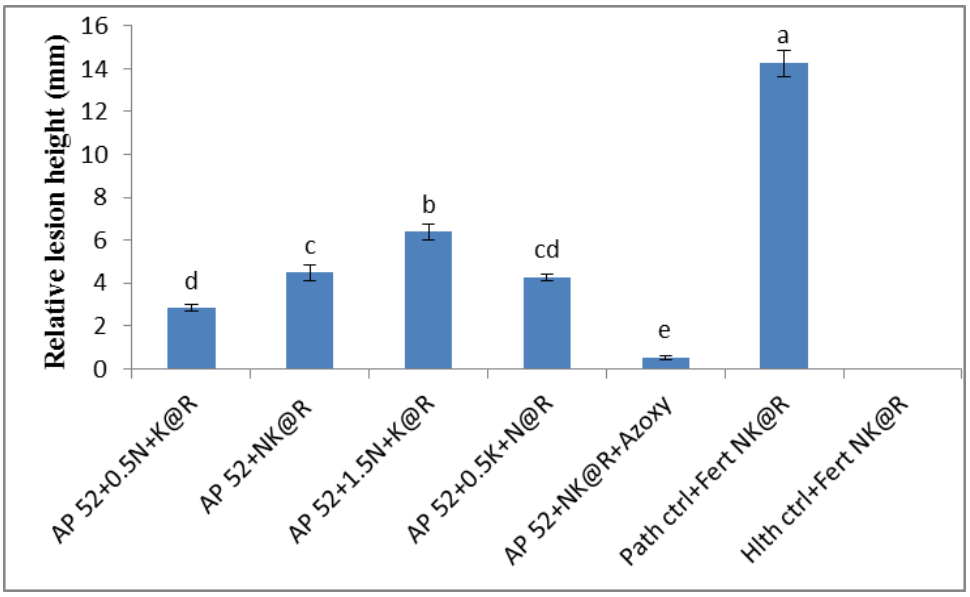

Figure 2. Mean total of percent relative lesion spread (mm) on rice harvested during summer alongside various treatments. The error bars shown represent ± S.E.

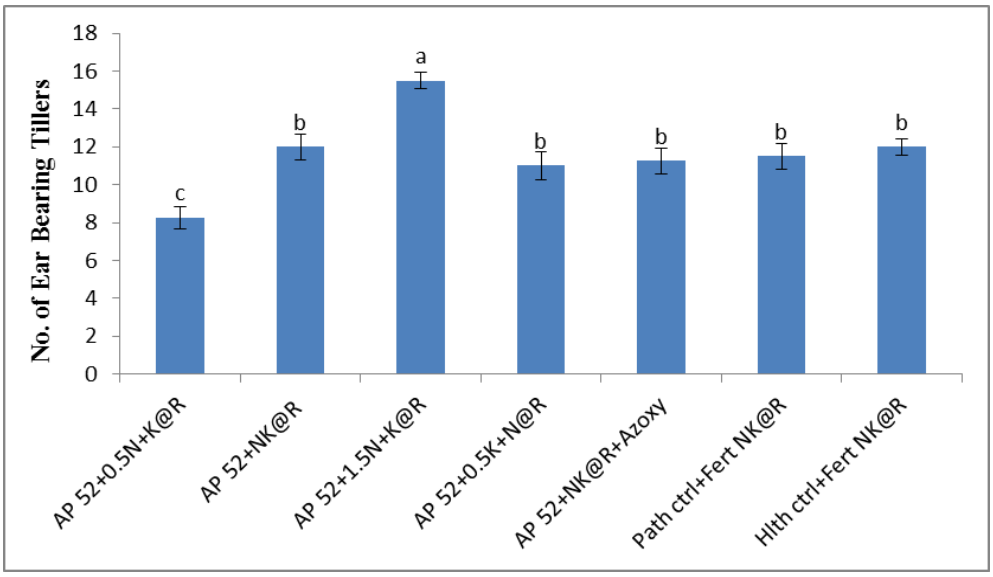

Figure 3. Mean total number of ear bearing tillers of rice harvested during summer, alongside various treatments. The error bars shown represent ± S.E.

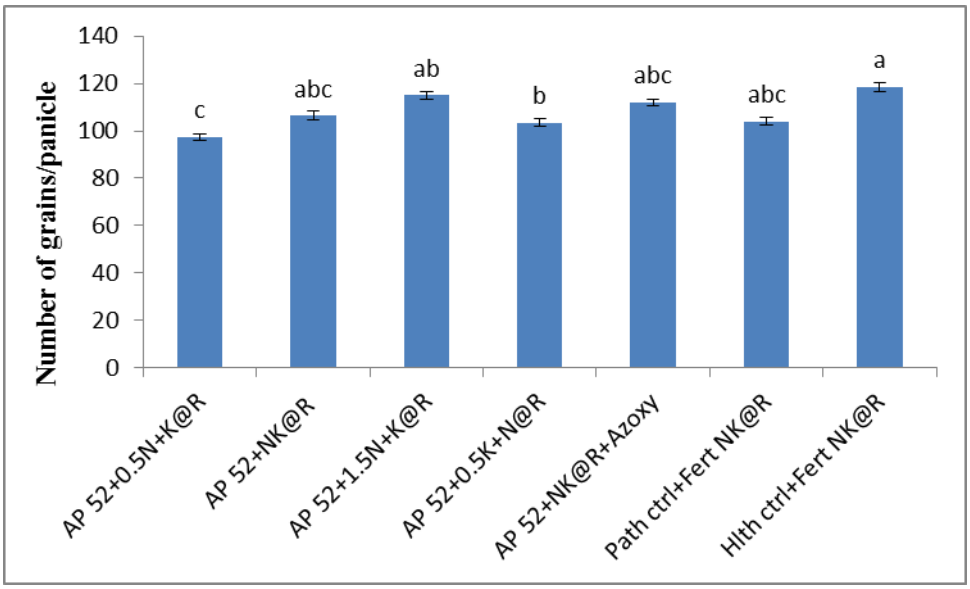

Figure 4. Mean total number of grains per panicle of rice harvested during summer, alongside various treatments. The error bars shown represent ± S.E.

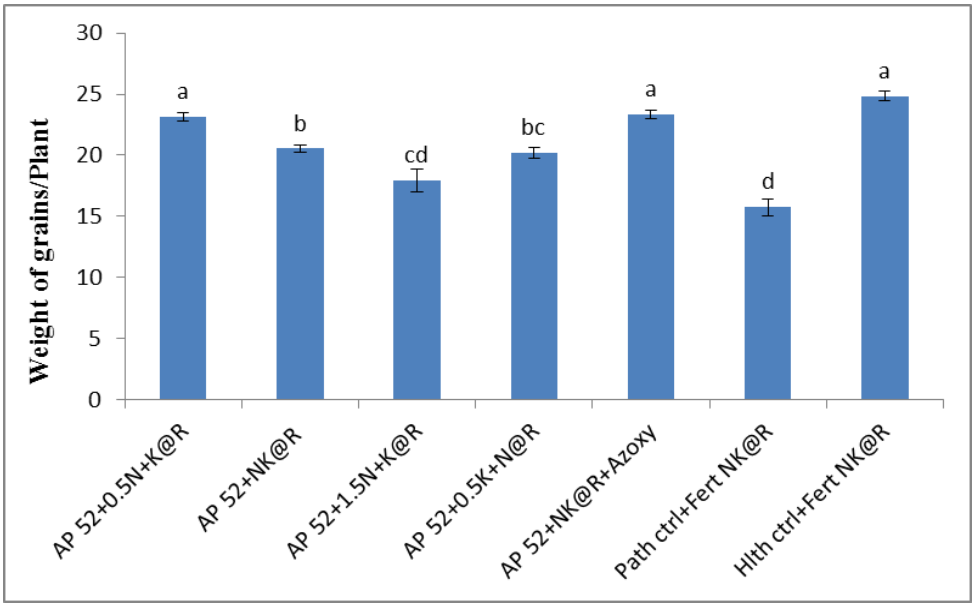

Figure 5. Mean total weight of grains per plant of rice harvested during summer, alongside various treatments. The error bars shown represent ± S.E.

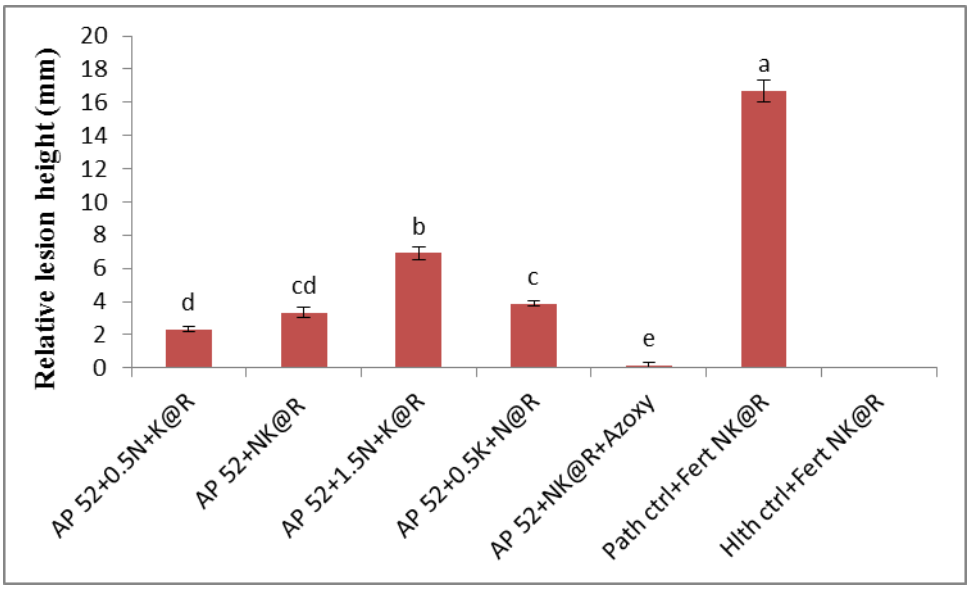

Figure 6. Mean total of percent relative lesion spread (mm) on rice harvested during fall season, alongside various treatments. The error bars shown represent ± S.E.

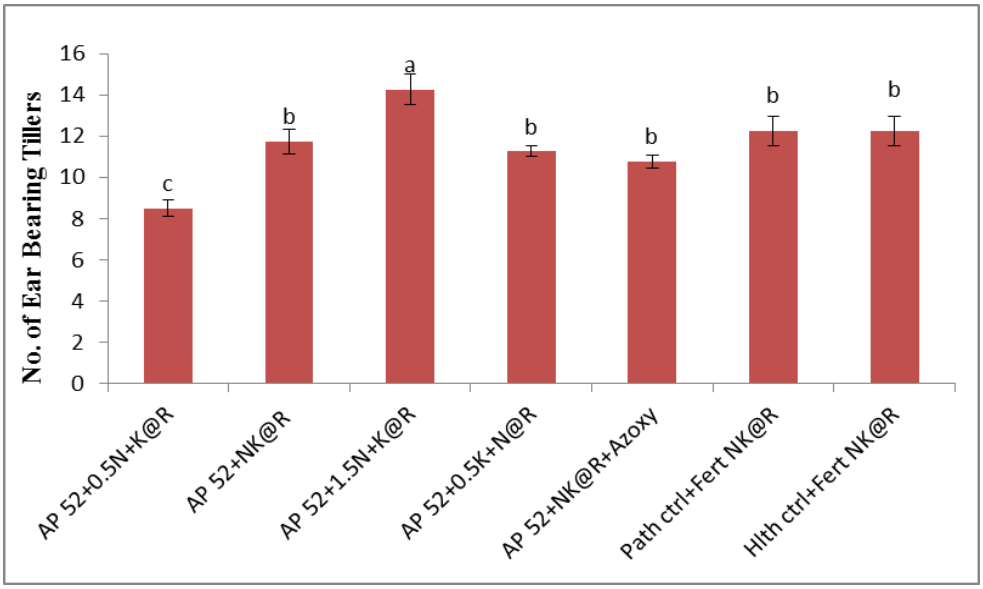

Figure 7. Mean total number of ear bearing tillers of rice harvested during fall season, alongside various treatments. The error bars shown represent ± S.E.

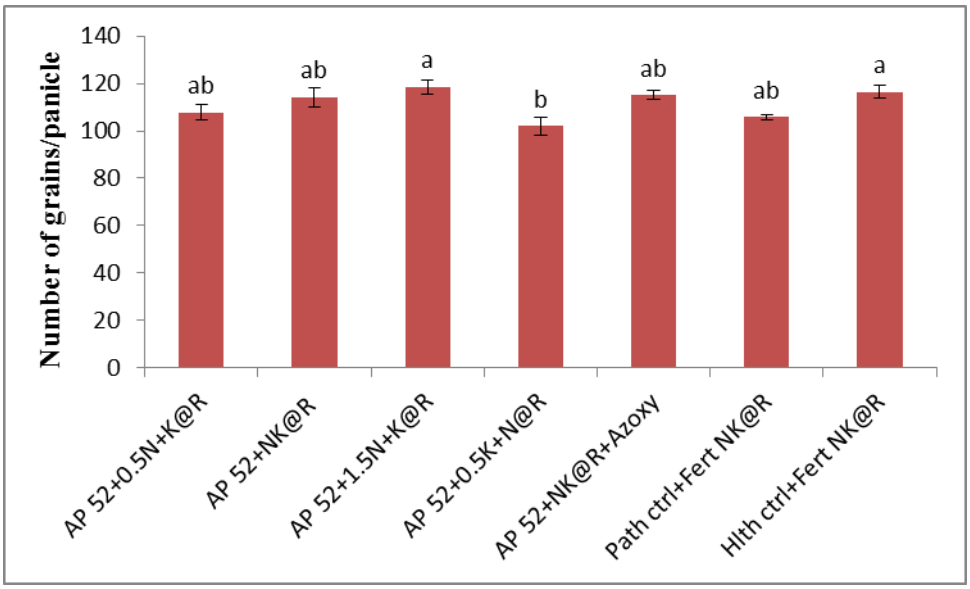

Figure 8. Mean total number of grains per panicle of rice harvested during fall season, alongside various treatments. The error bars shown represent ± S.E.

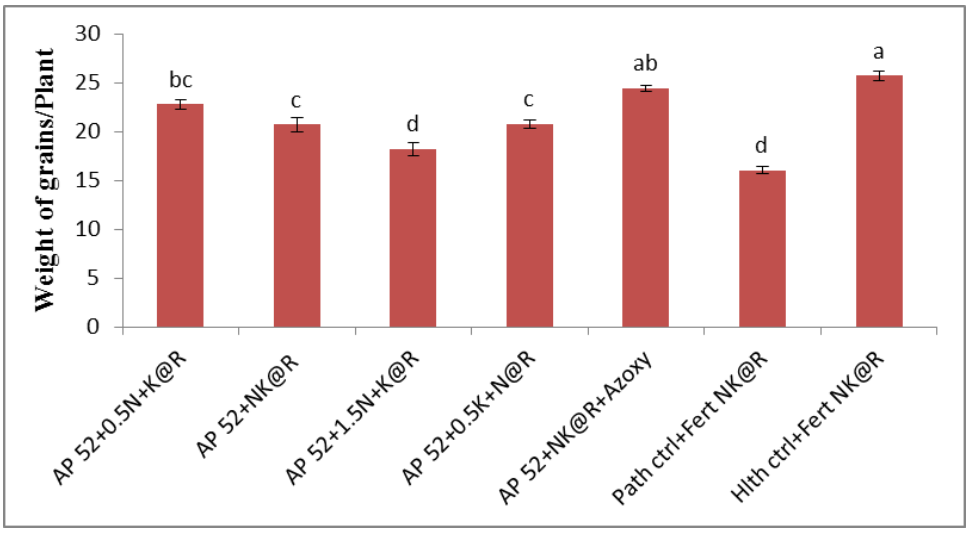

Figure 9. Mean total weight of grains per plant of rice harvested during fall season, alongside various treatments. The error bars shown represent ± S.E.

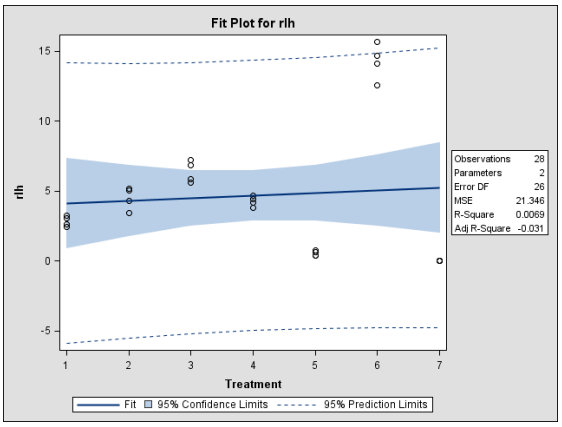

Figure 10a

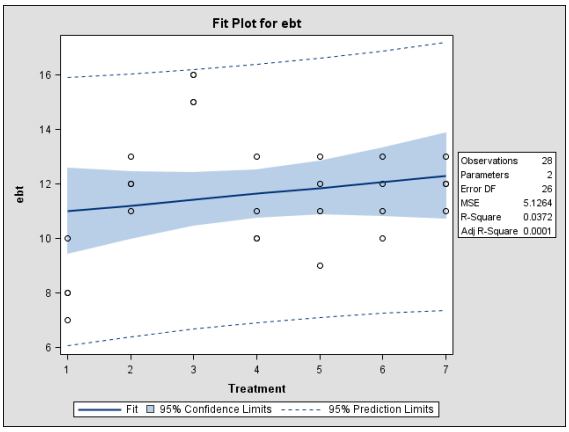

Figure 10b

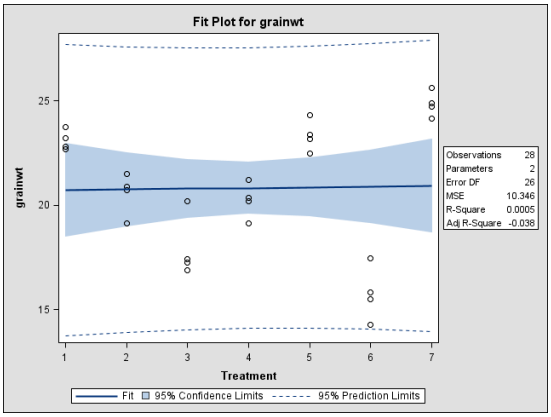

Figure 10c

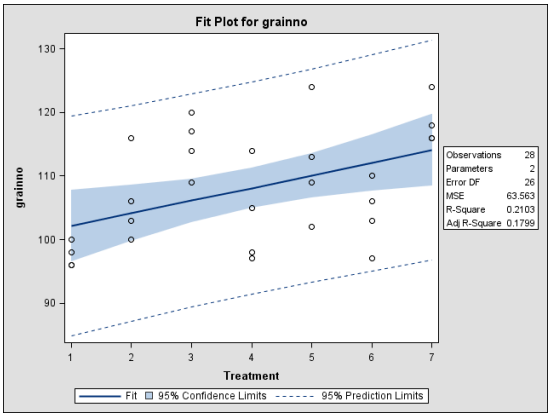

Figure 10d

Figure 10 (a through d). Linear regression model fit plot diagnostics for treatments compared with relative lesion height, number of ear bearing tillers (ebt), number of grains per panicle, and total grain weight per rice plant (Summer experiment).

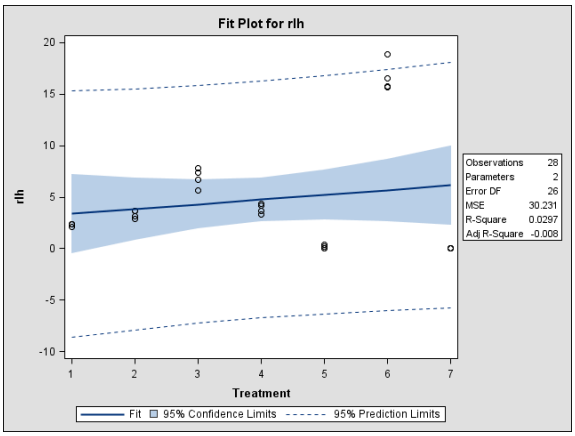

Figure 11a

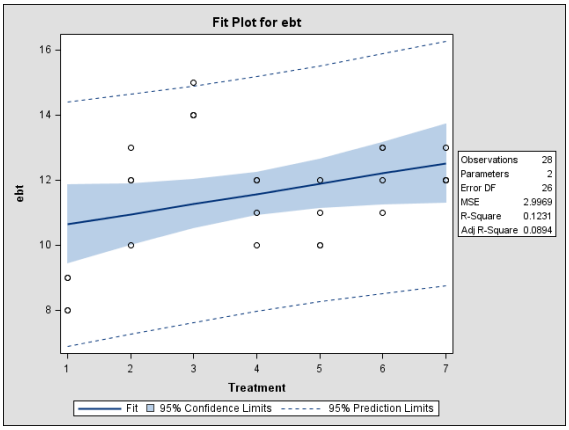

Figure 11b

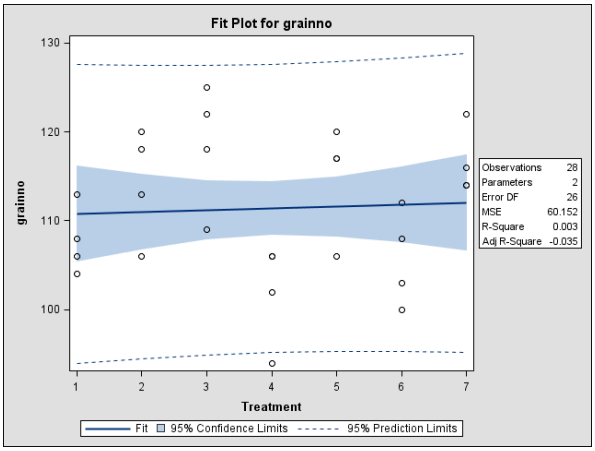

Figure 11c

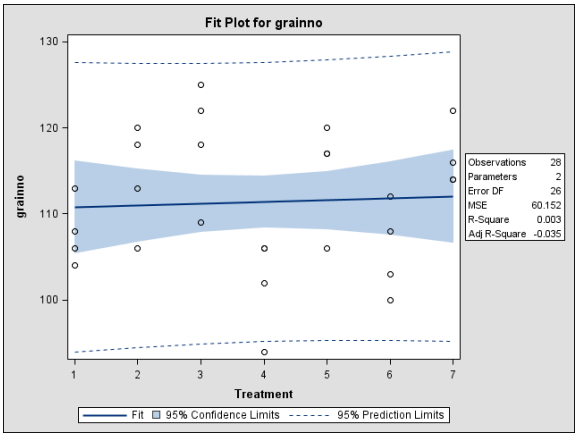

Figure 11d

Figure 11 (a through d). Linear regression model fit plot diagnostics for treatments compared with relative lesion height, number of ear bearing tillers (ebt), number of grains per panicle, and total grain weight per rice plant(Fall experiment).