

**Management of Sheath Blight and Enhancement of Growth and Yield of Rice with
Plant Growth-Promoting Rhizobacteria**

by

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Abstract

Sheath blight (ShB) of rice caused by *Rhizoctonia solani*, causes significant yield losses worldwide. Strong sources of genetic resistance are not available for ShB, and the disease is currently managed through use of chemical fungicides. Fungicidal management of ShB often gives inconsistent results and is not economical. Indiscriminate use of fungicides and chemical fertilizers to increase rice yields creates several concerns relating to environmental hazards, pathogen resistance, leaching losses, and destruction of beneficial microflora. Use of plant growth-promoting rhizobacteria (PGPR) as biocontrol agents is gaining popularity in managing rice diseases and in enhancing growth and grain yields. The objectives of this study were to 1) screen various PGPR strains for suppression of *R. solani*, and enhancement of rice seedlings vigor and select elite PGPR strains, 2) to evaluate the elite PGPR strains for suppression of ShB and for enhancement of growth and yield of rice under field conditions, and 3) to determine the mode of action of the elite strain for its disease suppressing and growth-promoting activities. Seventy PGPR strains with known activities on other crop-pathosystems were screened for *in vitro* antagonism against *R. solani* and for growth promotion of rice seedlings. The majority of the strains significantly suppressed the mycelial growth of pathogen, and improved rice seedling vigor and growth under *in vitro* conditions. Four strains completely inhibited sclerotial germination of *R. solani* under *in vitro* conditions. Of 70 strains, 31 strains significantly suppressed ShB lesions when tested in a detached leaf assay. Among these, one elite strain *Bacillus subtilis* MBI 600 was superior. Strain MBI 600 was produced in commercial proprietary

liquid formulation and designated as Integral[®] and tested for its growth promoting characters, and found to produce only siderophores and negative for chitinase, cellulase, HCN, IAA and phosphate solubilization. Integral was compatible to various commonly used fungicides such as propiconazole, validamycin, benomyl, tricyclazole, mancozeb, hexaconazole, carbendazim and azoxystrobin. Nursery and field trials were conducted in randomized block design with eight replications to assess the efficacy of Integral at A. P. Rice Research Institute, Maruteru, India during 2009 against ShB of rice CV. Swarna. Integral was applied as a seed treatment (ST), seedling root dip (SD) and foliar spray (FS) at concentrations of 2.2×10^8 and 2.2×10^9 cfu ml⁻¹. Seedling growth parameters and ShB severity were measured by calculating the highest relative lesion height (HRLH) at 90 days after transplanting. Seed bacterization with Integral resulted in enhanced root (9.3 to 14 cm) and shoot lengths (37 to 45 cm) over the control (8.4 and 36 cm, respectively) in the nursery. On a transplanted crop in the field, ShB severity was significantly lower when Integral was applied as ST + SD + FS at 2.2×10^9 cfu ml⁻¹ (19.2 to 26.5), followed by at 2.2×10^8 cfu ml⁻¹ (24.5 to 29.4) compared to the control (56.2 to 69.7). The ShB severity in carbendazim treated plants ranged from 16.8 to 19.8. Besides, the tiller production per plant was significantly higher in Integral treated plots at 2.2×10^9 cfu ml⁻¹ (12.3 to 12.9) compared to the control (10.0 to 10.5). Highest grain yields were recorded in Integral treated plots at 2.2×10^9 cfu ml⁻¹ (5922 to 6207 kg/ha) compared to the control (3925 to 4199 kg/ha). Scanning electron microscopy studies from an interaction between Integral and *R. solani* showed that Integral caused loss of structural integrity, maceration, shriveling, and reduction in hyphal width of *R. solani*. Deterioration of inner sclerotial filaments was observed when sclerotia were treated with Integral. Seed colonization studies showed that Integral was able to survive on rice seeds for up to six days following seed treatment. Integral seems to be a good root and rhizosphere colonizer.

Overall, Integral significantly reduced the ShB severity, and increased seedling vigor and grain yields in rice under field conditions and seems to have a potential for commercial application for rice ShB disease management.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Rice (*Oryza sativa* L.) is the most widely cultivated food crop in the world. Global rice production was approximately 680 million tons during the year 2009. Rice is being cultivated in 114 countries throughout the world, and more than 50 countries have a minimum annual production of 100,000 tons. The majority of the rice (90%) is being produced in Asian countries with China and India being the major producers (37). The other major rice producing countries are Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Philippines, Brazil, and Japan. In the United States, rice has been produced for 300 years and currently has an annual production of 9.2 million tons. Major rice producing states of the US include Arkansas, California, Louisiana, Mississippi, Missouri, and Texas.

Rice cultivation is often subjected to several biotic stresses of which diseases like blast, sheath blight (ShB), stem rot, and bacterial blight are important. ShB of rice is an important soil-borne fungal disease (*Rhizoctonia solani* Kuhn) causing up to 40% of yield losses annually, especially when susceptible cultivars were grown (89). The disease manifests initially as water soaked lesions on sheaths of lower leaves near water line. The dense crop canopy and high relative humidity (>95%) in the canopy usually favors the ShB development. As the disease advances; the lesions expand and are bleached with a brown border. Under ambient conditions, the disease assumes severe form and chaffiness of lower grains in the panicle is usually seen. The pathogen survives in the form of sclerotial bodies in the soil for several years, on stubbles of

the previous season's crop and on weeds (57, 58). Many chemical control methods are available in combating the disease and often ShB outbreaks are common. Effective management of ShB in rice is possible only when the pathogen is eliminated completely or the propagules are brought down below economic threshold limits at field level. Genetic sources of ShB resistance are not adequate, and present management strategies mostly involve use of chemical fungicides (85). The adverse effects of chemical fungicides on environment and beneficial microflora are evident and so an economic and viable alternative for ShB management is essential.

Biological control of plant pathogens though gaining popularity in majority of crops, its utilization in rice ecosystem is still at its infancy due to varied reasons. An effective biocontrol strategy of ShB is feasible only when the biocontrol agents survive, establish, proliferate and control ShB pathogen under conditions of crop submergence. Besides, the biocontrol agent should be able to induce systemic resistance thereby contributing to the disease control. Among different biocontrol agents, plant growth-promoting rhizobacteria (PGPR) are widely used in managing soil borne diseases of several field crops. The PGPR colonize the plant root systems through seed bacterization and show antagonism on soil-borne phytopathogens. Ability of these PGPR in plant growth-promotion and protection against soil-borne diseases further depends on many factors such as rhizosphere competence, persistence on seeds and plant roots, root colonizing capacity as well as synthesis and release of various metabolites. A successful bioagent against rice ShB should be able to control both the mycelial and sclerotial stages of pathogen, besides contributing to growth promotion and yield. Several bacterial strains were found to possess the ability to protect rice plants from blast, ShB, sheath rot and stem rot diseases (120). Of these, PGPR group offers an effective means of antagonism against ShB pathogen (69). Besides, these PGPR also contribute to enhanced seedling growth, induced systemic resistance

against diseases and thereby yield increase (87). Of different PGPR, fluorescent *Pseudomonads* and *Bacillus spp* group of bacteria offer an effective control of ShB besides inducing growth promoting effects and systemic resistance. Bacteria isolated from rice seeds and rice ecosystem was able to effectively suppress ShB besides producing growth promoting effects.

For PGPR to be effective under IDM, their chemical compatibility is essential (77), since rice production generally involve use of fungicidal mixtures with different ingredients (111). The present study was therefore aimed at identifying a potential PGPR strain in ShB management as an alternate or supplement to the existing chemical control. The objectives of investigation were to 1. Screen different PGPR and identify a potential strain effective against ShB disease and growth promotion in rice, 2. Determine the fungicidal compatibility of potential PGPR strain and to characterize the strain biochemically, 3. Evaluate the efficacy of potential PGPR strain in growth promotion and ShB management under greenhouse and field conditions, and 4. Determine its mode of action and colonization potential. The results obtained in the present study will be useful in devising strategies for an effective biocontrol based IDM strategy for ShB. The literature pertaining to ShB on rice is reviewed here under separate heads.

Etiology, Distribution and Spread. Rice ShB pathogen survives from one crop season to another through sclerotia and mycelia in plant debris and also through weed hosts in tropical environments (57). In temperate regions, the primary source of inoculum is sclerotia produced in previous rice crops (58). Both mycelia and sclerotia survive in infected plant debris. Mostly the survival is through sclerotia dropped in field during harvest, which will infect the crop during next season.

Changes in the magnitude and variability of temperature, precipitation and other climatic variables were found to have tremendous influence on plant diseases. ShB and blast diseases in

rice were found to be severe at elevated CO₂ concentrations (43). Areas under progress curves of disease severity and those of percent diseased rice tillers were positively correlated to the relative initial inoculum density of ShB pathogen. Further, rice yields were linearly and negatively correlated with disease severity and percent tillers affected (116). Further, rice diseases like ShB and bacterial blight were found to be prevalent in kharif (rainy season) (98). The vertical development of rice ShB is primarily dependent on the average daylight time within the first 5 days followed by the average RH and temperature (30). High temperatures and high humidity favor ShB lesion development both length wise and breadth wise in rice under laboratory conditions. Further, the lesion development was faster in sheaths inoculated with sclerotia than in already infected sheaths (102).

ShB disease severity was positively correlated with sandiness of soil. Further, the disease incidence was highest in wet soils with 50-60% water holding capacity (WHC) and lowest in submerged soils with 100% WHC (101). Infection on plants was very high when oil cakes was applied immediately after sowing; whereas its infection was low (compared to control) when oil cakes were applied at 20 days after sowing. The extent of damage of rice seedlings due to ShB incidence is dependent on resistance levels among the rice strains, average daily temperature, and frequency of rain. However, no significant relationship between incidence time and damage loss due to ShB was reported (28). Pot culture studies on the susceptibility of rice seedlings to *R. solani* inducing ShB disease revealed that disease incidence and development was rampant on 20- to 30-days-old rice seedlings compared to seedlings of 30- to 40-days-old under artificially inoculated conditions (27).

Isolation, pathogenicity and cross inoculation tests with ShB pathogen revealed that several host plants were found to be host plants to *R. solani*. Besides, several weeds like *Cyperus*

rotundus, *C. difformis*, *Cynodon dactylon*, *Echinochloa colonum*, *Setaria glauca* (*S. pumila*), *Panicum repens*, *Brachiaria*, *Commelina obliqua*, and *Amaranthus viridis* were identified as collateral hosts, and the pathogen perpetuates in these hosts in absence of rice plants (2). Studies on the role of seed borne inoculum in rice ShB disease development and observed no correlation between degree of seed discoloration and isolation frequency of pathogen. Further, the biocontrol studies with *Trichoderma harzianum*, *T. viride*, *T. virens* (*Gliocladium virens*) and *Pseudomonas fluorescens* and also with carbendazim proved that *R. solani* was internally seed-borne in nature. However, despite its good survival in seed, the transmission by seeds to rice plants under field conditions was very poor (109).

Pathogenicity of *Rhizoctonia solani*. Rice ShB symptom production under artificial conditions depends on the method of inoculation. Of different inoculation techniques such as single grain insertion, single sclerotium insertion, and mycelial suspension injection, single sclerotium insertion was most effective with highest ShB symptom (68.5 to 80.0%), lesion length (2.45 to 4.75 cm) and percent disease index (32.5-43.5) followed by single grain insertion technique (16). Maximum disease severity was observed when sheaths and leaves were inoculated with 7-day-old propagules of the pathogen (26).

The amount of *R. solani* inoculum plays a major role in uniform ShB disease development. Inoculum at the rate of 0.2 mg when placed inside the leaf sheath with a few drops of sterile water, induced single, discrete and uniform-sized lesions irrespective of the inoculum type (mature, immature sclerotium, and mycelium). Use of immature sclerotia is a simple, rapid, and highly reproducible disease production assay under greenhouse conditions (6). Further studies indicated that the pathogen when inoculated on inner surface of rice sheath initially colonized the surface before producing lobate, bulbous appressoria and infection cushions. The colonization of

epidermal and mesophyll cells was both intra-and inter-cellular, and the intra-cellular hyphae were thick and deformed whereas the surface hyphae from primary lesions penetrated the healthy tissue both by hyphal tips as well as branched lobate appressoria. Early infection on a healthy plant within 12 h is possible when mycelium of the pathogen was used instead of sclerotial bodies (7).

The ShB pathogen can infect the rice crop at any stage of growth from seedling to flowering by different inoculum sources. Among the different types of symptoms that are produced based on the source of inoculum and host growth stage, ShB is the most prominent and common one. Other symptoms on rice include pre - and post-emergence seedling blight, banded leaf blight, panicle infection, and spotted seed (1). Three pathogens are found to cause ShB disease in rice. They are *R. solani* (*Thanatephorus cucumeris*), *R. oryzae-sativae* (*Ceratobasidium oryzae-sativae*), and *R. oryzae* (*Waitea circinata*). Combined inoculation with these pathogens resulted in highest disease severity. Further, ShB incidence was maximum when treated with *R. solani*, moderate with *R. oryzae-sativae*, and low with *R. oryzae*. Results also indicated that *R. oryzae* was antagonistic to *R. solani* whereas *R. oryzae-sativae* did not show any antagonism towards *R. solani* (3).

Host range studies indicated that crop plants such as *Cajanus cajan*, *Capsicum annuum*, *Curcuma longa*, *Dolichos biflorus*, *Lycopersicon esculentum*, *Panicum miliaceum*, *Paspalum scrobiculatum*, *Setaria italica*, *Sorghum vulgare*, and *Zea mays* were moderately susceptible to the pathogen. The other plants such as *Brachiaria mutica*, *Cynodon dactylon*, *Cyperus rotundus*, *Echinochloa colona*, *Eleusine corocana*, and *Phaseolus aureus* were susceptible to the pathogen *R. solani*. The remaining host plants *Dolichos lablab* var. *typicus* and *Vigna sinensis* fall under the most susceptible category (75).

The rice ShB pathogen produces several cell wall degrading enzymes in improved Marcus medium under *in vitro* conditions. Immersion of rice sheaths in these enzymes resulted in breaking of callus, sheath cell, organelle, and also in cell wall cracking and mitochondrial damage (129). Cell wall degrading enzymes of the pathogen include polygalacturonase (PG), cellulase (Cx), pectin methylgalacturonase (PMG), and polygalacturonic acid trans-eliminase (pectate lyase) (PMTE) in improved Marcus's medium of which the activity of PG, Cx, and PMG were significantly higher than PGTE and PMTE. These CWDEs play an important role in lesion formation and expansion (18). The *R. solani* isolates that produce extra cellular cellulose, pectolytic and protease enzymes under *in vitro* conditions exhibited greater virulence over isolates devoid of enzyme production. All the isolates were obtained from areas which experienced full introduction of hybrid and high yielding rice varieties (99).

Rice ShB pathogen also produces toxin that induce characteristic symptoms on rice leaves, wilting of seedlings, and inhibited rice radical growth. A positive correlation was noted between crude toxin production and the virulence of the pathogen. The radicles and seedlings of resistant rice cultivars were more tolerant to the crude toxin compared to susceptible cultivars, indicating the scope of resistance screening through treatment of rice radicles with the crude toxin (125). Studies on pathogenicity factors of *R. solani* indicated that melanin producing cultures (M+ type) are more virulent than non melanin producing cultures (M- type) (56).

Sheath blight management. ShB disease is currently managed through a combination of different methods. However, ShB epidemics are still common in all crop growing areas of the world. The different ShB management strategies that are available are listed hereunder.

Host plant resistance. Presently, no strong genetic sources of resistance are reported against rice ShB. Resistance among the cultivable varieties in the southern United States currently

ranges only from very susceptible to moderately resistant. The yield losses were reported to be 8% in moderately resistant (cv. Jupiter) and up to 30% in very susceptible (cv. Trenasse) in rice fields with artificial inoculation (32). In a separate study during 2003 through 2005, following artificial inoculations with ShB pathogen, a significant increase in disease incidence and severity was observed in moderately susceptible and very susceptible cultivars. Further, a yield loss of 4% was noticed in moderately susceptible cv. Francis and 21% was found in very susceptible cv. Cocodrie. (33).

Several screening methods for determining ShB resistance are reported. Greenhouse inoculation with 2g of pathogen multiplied on rice grain and hull medium placed on soil surface around the plant is a reliable method for germplasm screening against ShB resistance. An effective and standard micro-chamber screening method in quantifying resistance to rice ShB was reported. Rice seedlings were inoculated at the three to four-leaf stage with PDA plugs containing mycelium and then covered with a 2- or 3-liter transparent plastic bottle for maintaining high humidity after inoculation. Consistent results were obtained and the resistance levels matched both under greenhouse and field conditions (44).

Chitinase production in rice cultivars is an important factor contributing to disease resistance against ShB. Greenhouse studies revealed chitinase activities in rice plants at 24h after inoculation of moderately resistant cultivars whereas in susceptible cultivars, the chitinase activity was detected after 36h. Western blot analysis revealed that chitinases were induced in plant system following *R. solani* infection and they were greater in moderately resistant rice cultivars with low ShB disease severity compared to susceptible cultivars (106). In a different study, 41 homozygous rice lines that were transformed with chitinase and beta-1, 3-glucanase genes for their resistance to ShB and it was observed that 92% of them were either moderately

resistant or moderately susceptible. A significant correlation was obtained between ShB resistance in resistant or susceptible transgenic lines with chitinase activity (63).

Induction of systemic resistance in rice plants against ShB is often in practice. Seed treatment with chemicals such as salicylic acid, acetylsalicylic acid, DL-gamma-amino-n-butyric acid, gamma-amino-butyric acid, amino-iso-butyric acid, indole-3-pyruvic acid, IAA, IBA, nicotinic acid, iso-nicotinic acid, DL-norvaline, propionic acid, benzoic acid, para-aminobenzoic acid, and zinc sulfate were proved effective in inducing systemic resistance to ShB. Among them, salicylic acid + gamma-amino-n-butyric acid treatment were the most effective in reducing lesion length over control (22).

Screening of rice germplasm is a continuous process to identify definite sources of resistance against ShB. Out of two hundred rice accessions representing 15 *Oryza* species that were screened for major rice diseases, seven accessions, IRGC 81940 and 81941 (belonging to *O. nivara*) and IRGC 103303, 105165, 105268, 105270, and 105272 belonging to *O. australiensis* were resistant/moderately resistant to ShB and sheath rot. The IRGC 105272 of *O. australiensis* was found to be resistant to ShB, sheath rot, and bacterial blight diseases. These accessions can serve as donors of multiple disease resistance in an irrigated agroecosystem for widening the resistance gene pool of *O. sativa* (96). A new rice cv. NDR2030 derived from the cross Ratna/Saket//IR36, which is a mid-early cultivar with high yield potential and long-slender, translucent grains was reported. The cultivar is resistant to gall midge, moderately resistant to white-backed planthopper, gall midge biotype 2, bacterial leaf blight and ShB (121). In another study, a new rice variety Giri (IR36 X Bhasamani) was released for low land rice cultivation in 2002 by the State Variety Release Committee for West Bengal, India. The variety was found resistant to ShB, bacterial blight and tolerant to submergence (108).

Agronomic practices. Incidence of ShB in rice fields is dependent on the method of planting and plant population density. Investigations at farmers' fields and experimental fields (Taizhou institute of agricultural science, China) revealed that square method of transplantation resulted in optimum high-yield density, higher leaf area index and dry matter production. This method of transplantation also contributed to increased ShB resistance and higher grain yields (127). In a separate study, it was reported that sparse planting resulted in lower ShB occurrence and greater lodging resistance in rice. The other important effects of sparse planting included fewer number of stems/m², more stems/hill, delay in date of maximum tillering stage, heading time, ripening time, greater number of pods per head and more pods on secondary rachis-branches (112). Planting of rice seedlings far from the bund resulted in reduced ShB incidence since bunds have weed hosts of *R. solani*. Both vertical and horizontal spread of the disease in the field increased from the source of infection and with the increase of plant age (103). Submergence of the crop had a negative effect on disease progress and resulted in reduced ShB disease development (24). Maximum survival of ShB pathogen was reported in 50% soil saturation whereas maximum survival of fungal bio-agents like *Trichoderma viride* and *T. harzianum* was reported at 100% soil saturation. Control of ShB as well as increase in plant growth by these bioagents was effective under submerged conditions (10).

Soil amendment with organic fertilizers has a definite role in managing rice diseases. Organic fertilization with both animal manures and composts resulted in enhanced growth and yield of rice. Besides, the incidence of rice diseases like ShB, blast and pests like brown plant hopper, stem borer and leaf folder are reduced remarkably (70). Of various soil organic amendments (*Azadirachta indica*, *Pongamia pinnata*, *Gliricidia maculata*, *Chromolaena odorata*, *Prosopis juliflora*, and *Terminalia bellirica*), *A. indica* at 150 kg/ha as oil cake was

most effective in reducing the ShB incidence (66.35% reduction over control), followed by *G. maculata* (as leaves), *Pongamia pinnata* (as oilcake), and *P. juliflora* (as leaves). Yield levels were significantly enhanced with soil amendments, and the greatest increase in yield was obtained with *A. indica* oilseed cake (3200.60 kg/ha vs. 2200.72 kg/ha for the control) (59). Low population densities of ShB pathogen were observed in rice fields amended with mustard and groundnut oilcakes. Further, the population densities of fungal antagonists such as *Aspergillus* spp and *Penicillium* spp were increased in amended soil. Other beneficial effects include congenial conditions for multiplication of fungal bioagent, *T. harzianum* and thickening, swelling and lysis of ShB pathogen hyphae due to bacterial activity (100). Greenhouse indicated that the bioagent *T. harzianum* was highly effective when the soil is amended with neem cake. Further, the ShB disease incidence and severity was less in clay loam soils compared to sandy loams (51). Application of 50% organics (as decomposed rice straw) in combination with 50% inorganic fertilizers increased rice yields by 23% and also harbored higher microbial communities over control and for plots that received 100% inorganic fertilizers. Further, the ShB incidence was delayed and the beneficial *Trichoderma* spp was higher in plots that received 100% organic manures when compared to application of 100% inorganic fertilizers (71). Pot and field studies on the effects of organic soil amendments in rice revealed that the mean soil fungal and bacterial population increased by 2 weeks and 10 weeks after addition of soil amendments. However, the populations of both fungi and bacteria decreased at 14 weeks after addition of soil amendments. Even the population levels of the ShB pathogen *R. solani* showed a steady decline by 2 and 10 weeks after addition of soil amendments but no significant reduction in ShB pathogen was observed at 14 weeks after addition (115).

Inorganic nutrient management is also a major factor determining rice ShB disease. Detailed investigations on comparative studies between plots under site-specific nutrient management (SSNM) and farmer's field practices (FFP) in rice during wet seasons of 1998 and 1999 across China, Vietnam, Philippines, Indonesia and India revealed that ShB and leaf folder are major N-dependent variables whereas ShB, grain discoloration, brown spot, and red stripe were major yield reducing factors (35). Plant variety and nitrogen fertilizers are the major factors influencing ShB disease and concomitant yield losses in rice, both during wet and dry seasons. Varieties with taller stature, fewer tillers, and lower leaf N concentration, such as IR68284H, generally had lower ShB lesion height, ShB index, and consequently lower yield loss from the disease. Disease intensity and yield loss increased with increasing N rates, but the magnitude of yield loss varied among varieties (119).

Among different plant nutrients, silicon (Si) plays an important role in imparting resistance against blast, brown spot, and ShB diseases of rice. The Si mediated resistance is due to a mechanical barrier caused by its polymerization *in planta*, accumulation of phenolics and phytoalexins, and activation of some pathogenesis-related proteins. Further, the prevalence of these diseases is more severe in rice grown in Si depleted soils (97). Field studies indicated that application of complete silicon fertilizer (mixture of silicon, nitrogen, phosphorus and potassium) and organic fertilizers increased early rice yields by 12 and 21%, late rice yields by 8 and 29% respectively. Besides, the incidences of rice diseases such as blast, ShB and stem borer were reduced significantly (122).

Plant extracts. The use of botanicals in the management of rice ShB is gaining importance of late. Different plant extracts are being used all over the world and among them, neem formulations are very effective in controlling the ShB incidence as well as in increasing grain

yields. Field application of neem formulations, 0.03% (300 ppm azadirachtin) and 0.15% EC (1500 ppm azadirachtin) @4.5 ml/L during afternoon hrs was very effective in reducing ShB incidence as well as in increasing grain yields (15). Greenhouse studies on the efficacy of neem products against rice ShB revealed that neem oil, its saturated fraction and its stabilized formulations were effective in containing the disease incidence as well as in reduction of percent infected tillers (39).

Besides, certain plant extracts such as *Odiyana wodier*, *Lawsonia alba*, *Ocimum sanctum*, and *Pongamia glabra* were found to be effective both in reducing the mycelial growth (70 to 85% inhibition) and sporulation of *R. solani* under *in vitro* conditions. Further, field studies with *O. wodier* and *O. sanctum* were very effective (26 and 28% ShB severity) over control (42%) (47). The plant extract of *Gaultheria* spp formulated as Biotos was found to be highly effective at 0.25% concentration and was superior both in controlling ShB severity (9.7%) and in increasing grain yields (9859 kg/ha). Further, the efficacy of Biotos was significantly superior over neem-based botanicals such as Achook and Tricure (13). Other plant extracts that are effective against rice ShB include *Allium sativum*, *Prosopis juliflora*, *Gynandropsis pentaphylla*, *Leucos aspera*, and *Vitex negundo*. (113). Palmarosa oil (@ 0.05 and 0.1% concentrations) effectively inhibited the mycelial growth and sclerotial production of rice ShB pathogen (74)

Leaf extracts of certain plant species were also used for effective management of rice ShB. Among them, the leaf extract of *Pithecellobium dulce* was highly effective in inhibiting mycelial growth of test pathogen (2.5 cm over 8.9 cm in control). Both the leaf extracts of *P. dulce* and *Prosopis juliflora* were equally effective in inhibiting sclerotial number, dry weight, and germination of the pathogen and also in controlling ShB with a disease incidence of 32.3 and 33.3%, respectively, over 76.2% in control (76). The leaf extracts of *Clerodendron viscosum*,

Lantana camara, and *Vitex negundo* were highly inhibitory to both mycelial growth and sclerotial production of *R. solani*, whereas the other tested leaf extracts like *Citrus aurantiifolia* and fenugreek were not effective (107).

Fungicides. Presently, ShB disease management is majorly achieved through systemic fungicides and also with certain non-systemic fungicides (85). The resistance gain by pathogen to these systemic fungicides is of concern, thus demanding an evolution of newer fungicides and screening of certain commonly used fungicides before evolving a comprehensive and compatible integrated disease management (IDM). Moreover, host plant resistance to ShB range only from very susceptible to moderately susceptible levels in rice (33), thus chemical management has become a necessary component for an effective IDM.

Application of fungicidal mixtures and pesticides for the control of pests and diseases is common in rice. The compatibility of these chemicals is a pre-requisite for effective management of these biotic stresses. Plant hopper is an economically important pest and the general practice is to target both ShB disease and plant hoppers in rice at a time. Field studies were carried out during rainy and post rainy seasons of 2005 and 2006 on pesticidal compatibility against rice pest and disease management. Results indicated that combined application of the insecticide imidacloprid (Confidor 200 SL) at 0.25ml/L and the fungicide validamycin (Rhizocin 3L) at 2.5 ml/L were high compatible and effective in reducing plant hopper and ShB incidence besides contributing to yield increase (11). Fungicidal combinations are popular in management of rice diseases in general and ShB in particular. Greenhouse and field studies with the fungicide Lustre (37.5SE) (flusilazole + carbendazim) against ShB revealed that application of the triazole mix could reduce disease severity and increase yields. Further, it was proved that the test fungicide

was a safe combination fungicide without any phytotoxic symptoms. Its prophylactic application gave better results than as a curative application (94).

Use of fungicides with a broad spectrum of activity against more than one disease is common in rice. Apart from blast, ShB, sheath rot and brown spot are the major economic diseases and a broad spectrum fungicide against all these rice diseases is economical. Among different fungicides screened under laboratory and field conditions (from 2002 to 2004), Tilt 25 EC (propiconazole) at 0.1% was highly effective against all these diseases. Whereas, Bavistin 50 WP (carbendazim) and Contaf 5 EC (hexaconazole) at 0.1% concentration were effective against ShB and sheath rot. Among other fungicides, Rhizocin 3 L (validamycin) at 0.25% was effective against ShB. Laboratory studies revealed that Tilt 25EC followed by Contaf 5EC were effective against all the test pathogens (68). In a separate study on the evaluation of seed treatment against rice diseases, Vitavax 200 (carboxin + thiram) application (0.3% of seed weight) reduced the incidence of brown spot, blast, bakanae, foot rot and seedling blight in seed beds. Brown spot, narrow brown spot, blast, ShB and sheath rot diseases are the diseases that are controlled in transplanted fields. Highest weight of healthy seeds per panicle (17.5g), highest number of healthy seeds per panicle (158.6), and highest seed yield (18.07% increase over control) were recorded in Vitavax 200-treated seeds (45).

The effective fungicides against rice ShB at field level are Akonazole 250 EC (propiconazole) and Folicur EW 250 (tebuconazole) in reducing percent tiller infection, relative lesion height, and percent disease index (PDI) over control. Besides, a significant improvement in grain yields was reported with these fungicides (79). The fungicide Monceren (pencycuron 250 SC) was also effective against ShB at field level both in terms of disease reduction as well as increase of grain yields. Other effective fungicides include RIL 010/F1 25 SC, RIL 010/F1 50

SC, Rhizolex 50 WP, Rhizocin 3L, Folicur (tebuconazole) 250 EW, Contaf (hexaconazole) 5 EC, and Tilt (propiconazole) 25 EC at higher concentrations and were equal with Bavistin (carbendazim) 50 WP. Shield (clopyralid) 2.62 SC was the least effective one (67). Field application of Tilt/Result (propiconazole) at 0.10% as sprays twice was effective in reducing ShB severity and improving grain yield over others (12).

Certain new fungicidal formulations were also found effective against rice ShB. Among them, Amistar 25 SC @ 1.0 ml L⁻¹ (30.6%) and RIL-010/FI 25 SC at 0.75 ml L⁻¹ (30.1%) showed a high degree of efficacy in reducing the disease severity and were superior over the standard fungicides (validamycin at 2.5 ml L⁻¹). Highest grain yields were also reported in these fungicide treatments (93). The fungicide pencycuron (Monceren 250 SC) was most effective when sprayed at 35 and 55 days after transplanting. A ShB disease severity of 2.7 and 4.7% was observed after its first and second application in successive years of study during 2001-'02 (20). Strobilurins are new group of fungicides that are showing promising results in rice ShB disease control. The biofungicidal activity of strobilurins was reviewed and comparisons were drawn between its efficacy and the existing recommendations against ShB such as carbendazim, validamycin, and other triazoles. Results indicated that strobilurins were very effective both in terms of disease reduction as well as in increasing grain yields (14). Studies on the respiratory activity of metominostrobin against ShB pathogen and concluded that mycelial cells of pathogen induce an alternate respiratory pathway in response to blockage of cytochrome pathway. However, the alternate pathway of the pathogen could also be suppressed by some flavonoids, suggesting that metominostrobin is to be used in conjunction with plant components especially when the fungicide is applied in a prophylactic manner (36).

The other new fungicidal formulations that show promising activity against rice *R. solani* include O, O-diaryl O-ethyl phosphorothionate compounds (BG-8, BG-11, BG-14 and BG-19). These compounds when tested at 250, 500, and 1000 ppm under *in vitro* conditions against *R. solani* were found effective. Complete control of the pathogen was attained with application of BG-8 and BG-19 at 500 and 1000 ppm (40). In a separate study, the fungicides G/FT-3 (O, O-di (2, 4, 5-trichlorophenyl)-S-methyl phosphorothionate) and G/FT-9 (O, O-di (2, 4, 6-trichlorophenyl)-O-methyl phosphorothionate) were found to inhibit the mycelial growth of *R. solani* under *in vitro* conditions at 25-50 and 1-2 ppm respectively. *In vivo* studies on rice cv. Pusa Basmati-1 revealed that G/FT-3 and G/FT-9 caused ShB disease reduction of 52.8% and 43.9% at 100 and 4 ppm respectively (41).

Biological control. Biological control of plant pathogens though gaining popularity in majority of crops, its utilization in rice ecosystem is still at its infancy due to varied reasons. Rice, being a crop that is grown under inundated conditions; the survival, growth and establishment of biological control agents is questionable. However, effective management strategy of ShB disease is feasible only when the biocontrol agents those are in vogue in rice based cropping systems survive, establish, proliferate and control ShB pathogen and also have a synergistic growth promoting effect on the crop. Besides, the biocontrol agent should be able to induce systemic resistance thereby contributing to the disease control.

Fungal bioagents. Among the fungal antagonists, *Trichoderma* spp and *Gliocladium* spp are widely used in the management of rice ShB disease. These fungal antagonists are either applied to rice seed, soil, root dip and foliar spray for managing the disease. In pot culture studies, seed treatment of the bioagent *T. viride* resulted in ShB disease reduction. Further, the efficacy of *T. viride* was comparatively more than the bacterial bioagent *Bacillus subtilis* (25). Foliar

application of *Trichoderma* spp also was found to be very effective in reducing ShB severity. Studies on field application of *T. harzianum* as talc + CMC based formulation proved that disease severity was reduced by 52%. The bioagent was found effective when applied at 7 days compared to simultaneous application with ShB pathogen (52). The optimum dosage of the bioagent was found to be 4 or 8 g/L and increased grain yields were also reported (55). Spray application of the bioagent was highly effective on rice seedlings that received 60 kg N + 60 kg P + 40 kg K/ha (30 kg N and whole of P and K as basal and remaining 30 kg N at 20 and 40 days after transplanting) both in terms of reduction in ShB incidence, severity, and increased yields (53). Further, it was determined that the rice leaf isolate of *Trichoderma* spp was more effective compared to the rhizosphere isolate of *T. virens* (50). Soil application of *T. harzianum* was not effective both under greenhouse and field conditions. On the other hand, mixed mode of application of bioagent as soil treatment, root dipping, and foliar spray was found to be very effective in reducing ShB severity over control. However, foliar application of the bioagent alone was also effective under field conditions (62). Application of *T. viride* as root dip + spray was effective in reducing ShB severity by 59% under field conditions (81).

Combined applications of bioagents also were proved effective in controlling ShB both under greenhouse and field conditions. Combined applications of *T. viride* and *P. fluorescens* was effective without any negative effects in reducing rice ShB besides increasing number of productive tillers, higher grain and straw yields. However, individual applications of bacterial and fungal antagonists separately had more beneficial effects (72). Combined application of *G. virens* and *B. subtilis* was more effective in ShB disease reduction (73%) over their individual applications. Further, lower doses of bioagents ((2.5g/kg of *G. virens* and 10^8 cells/ml of *B. subtilis*) were necessitated in combined application compared to their individual applications

(104). Studies on cellulose activity of *Trichoderma* spp indicated that *T. hamatum*, *T. aureoviride* and *G. virens* were effective against rice ShB pathogen. Field studies indicated that the fungal bioagents exhibited good antagonism, and a ShB disease control effect of 32% was obtained with fungal antagonist mixture besides positive effects on seed setting rate and 1000-grain weight of rice plants (118). Evaluation of 800 strains of *Trichoderma* spp against rice ShB pathogen indicated that six strains were highly inhibitory to the growth of pathogen in dual culture studies. Among them, T3 was found superior in reducing the pathogen growth by 53% (117). Effective ShB disease suppression at field level can be obtained by soil application of *T. harzianum* and *T. viride* at a pH range of 5.1 to 6.0. A concomitant increase in plant growth and yield was obtained. Further, it was reported that population levels of *Trichoderma* spp are high and that of *R. solani* are low in acid soils (9).

Among other fungal bioagents that are effective against rice ShB, *Helminthosporium gramineum* is an important one. The culture filtrates and crude toxin of *H. gramineum* were highly inhibitory to *in vitro* growth of *R. solani*. The biologically active metabolite of the crude toxin is identified as “ophiobolin A” by spectroscopic analysis and was found to significantly inhibit the mycelial growth of *R. solani* at all concentrations tested. Field studies indicated that the crude toxin was highly effective in reducing the rice ShB disease incidence and severity without any adverse effects on growth and yield attributes (29). Application of avirulent strains of ShB pathogen was also found effective. Field studies during T. Aus and T. Aman of 2004 at BRRI, Gazipur, Bangladesh with three avirulent strains of *R. oryzae* (*Waitea circinata*) isolates on rice cultivar Swarna revealed that ShB incidence was low in terms of relative lesion height percentage, tiller infection percentage, and severity index when the inocula of the bioagent, *R.*

oryzae (isolate no.545) were broadcasted to the field at five days after inoculation with *R. solani* pathogen (4).

Bacterial bioagents. Among the bacterial biocontrol agents, PGPR offer a promising means of controlling plant diseases besides contributing to the plant resistance, growth and yield in rice (78). Of different PGPR, fluorescent *Pseudomonads* and *Bacillus spp* group of bacteria offer an effective control of ShB besides inducing growth promoting effects and systemic resistance. Bacteria isolated from rice seeds and rice ecosystem was able to effectively suppress ShB besides producing growth promoting effects. Further, these bacterial antagonists should be applied only after maximum tillering stage of the crop since ShB pathogen is rarely rampant during flooded conditions (61). Seed treatment with these antagonistic bacteria resulted in increased root and shoot length of seedlings. Foliar sprays with these antagonists resulted in reduced ShB incidence (105). The antagonistic activity of endophytic and epiphytic bacterial strains isolated from healthy rice seeds was studied against rice ShB pathogen. The strains S-11, S-13, S-14 and S-18 effectively inhibited mycelial growth of pathogen. Field application of the strain S-18 at 3×10^9 cfu /ml resulted in reduced ShB incidence (128). Marine bacteria also have antagonistic abilities to control different plant pathogens. Strains of marine bacteria isolated from sea mud and water of Lianyungang sea area of China were found effective. The bacterium has ability to inhibit plant pathogens like *Alternaria brassicae*, *Magnaporthe grisea* and *Botrytis cineria*. Further, the extracellular substance of the bacterium has good ShB controlling efficacy in pot and field experiments (84).

Strains of *P. fluorescens* were found to inhibit the rice ShB pathogen under *in vitro* conditions. All the strains of the bioagent (biovar 2) produced siderophores on King's B media. The volatile metabolites, extra cellular secretions and antibiotics of these isolates were inhibitory

to *R. solani*. All the antagonists could reduce germination and caused lysis of sclerotial bodies (48). The population densities of the strains were increased on rice root system (49). Rhizosphere isolates of *P. fluorescens* (PF-3 and PF-4) were also inhibitory to chilli damping-off pathogen *Pythium* spp. These isolates also produce salicylic acid, siderophores and hydrogen cyanide (HCN) that are responsible for inhibition of test pathogens (80). Besides, some rhizosphere isolates of *P. fluorescens* (PfMDU2 strain) also produce β 1, 3-glucanase. A significant relationship between the antagonistic activity of the bacterium against *R. solani* and its level of β 1, 3-glucanase, salicylic acid and HCN production was noticed (82). Rhizosphere isolates of *P. fluorescens* (GR1, GR25, GR27, WR49, WR55, and WR62) from chick pea and wheat crops were also inhibitory to mycelial growth of rice ShB pathogen up to 23 mm and with an inhibition zone of 12 mm. Even the sclerotial bodies of the rice pathogen were inactivated completely when they were pretreated with bacterial cell suspensions for 1 minute to 4 weeks (86). The isolates of *P. fluorescens* were found to be compatible with one another under *in-vitro* conditions. Strains of *Pseudomonas*, Pf7-14 (natural resistant to nalidixic acid) and P13-R (spontaneous rifampicin resistant mutants of P13) those were highly antagonistic to rice *R. solani*. are compatible with each other under *in vitro* conditions (66).

Efficacy of *P. fluorescens* strains in greenhouse and field conditions depend on time of application. Field studies indicated that spraying of *P. fluorescens* at 7 days before pathogen inoculation resulted in maximum reduction in ShB severity (59.6-64.4%) over simultaneous application and at 7 days after inoculation. Further, with inoculation at 7 days before pathogen inoculation, an increase in 1000-grain weight (27.2-29.5%) was reported (91). The *Pseudomonas* treated rice plants show increased chitinase activity at 2 days after inoculation. This increased induction of pathogenesis-related chitinase is attributed to its role in suppressing ShB disease

incidence and development (90). The optimum spraying time of the bioagent was during the first day of inoculation of the test pathogen on rice plants (95).

The mode of application of *Pseudomonas* spp also determines their efficacy in controlling ShB disease. The biocontrol effect of *P. aeruginosa* on *R. solani* was due to crude extracts of the bacterium and is turn dependent on the concentration of extracts and the treatment time. The duration of colonization of the antagonist on rice plants is directly related to the initial concentration applied (95). ShB in rice fields could be effectively controlled with foliar sprays of *P. fluorescens*. Increased grain yields and 1000-grain weight are also reported with foliar application of the bioagent (Pfr1) (92). In contrast, it was reported that seed coating of *P. fluorescens* (B41) was found to be comparatively more effective than soil drenching and foliar sprays in reducing ShB disease in rice under greenhouse conditions. However, field studies indicated that the bioagent was highly effective when applied as seed coating, soil drenching, and as seed coating + foliar sprays (with 10.5, 11.75 and 18.75% disease intensity, respectively, against 52% in control plots) (48). Dual treatment of *Pseudomonas* strain GRP3 as seed bacterization followed by root dipping resulted in inhibition of mycelial growth and sclerotial germination of *R. solani*. The ShB lesion length was reduced up to 46%. The results were significantly superior compared to single application methods of the bioagent and control. Additionally, the peroxidase activity and phenol levels in dual treated plots were higher in plants treated with GRP₃ compared to the control. This increase is attributed to the control by bacterial bioagent that induces systemic resistance in host plants (87).

Enhanced efficacy of *Pseudomonas* spp was reported against ShB disease when the bioagents are used in conjunction with other bacterial and fungal bioagents. Combined applications of *P. fluorescens* with *T. viride* were found to be effective in rice ShB control as well as in promoting

seedling growth (73). Talc based formulations of two *P. fluorescens* strains (PF1 and PF7) when applied through seed, root, soil and foliar sprays significantly reduced ShB and leaffolder incidence under greenhouse and field conditions. The bacterial mixture performed better than individual strains, showing a reduction of 62% ShB and 47 to 56% leaffolder incidence (21). In a separate study, it was reported that PGPR strains of *P. fluorescens* (PF1, FP7, and PB2) when applied in combination as bacterial suspension or as talc based formulations through seed, root, foliar, and soil application significantly reduced the ShB incidence (45%) under greenhouse and field conditions over their individual applications (29% reduction). A significant increase in yield was obtained with application of bioagent mixture over their individual applications (25.9% and 17.7% increase respectively over control) (83).

Bacillus spp are important gram positive PGPR in the biocontrol of rice ShB disease. The bacterium produces endospores and microscopic studies revealed that isolates of *B. subtilis* and *B. megaterium* exhibited effective inhibition against the pathogens of ShB and bakane diseases of rice (69). The fermented product of *Bacillus* strain Drt-11 was highly antagonistic to rice ShB pathogen, causing reduced sclerotial germination (40-60% inhibition over control), reduced hyphal growth and colony diameter (by 14%) besides increased rice seedling growth (17). The bacterial antagonist (*B. subtilis* A30) produces an antagonistic substance named P1 which is both thermostable and proteinase-stable one. Further, the antagonistic substance had a negative ninhydrin reaction and positive ninhydrin and biuret reactions after acid hydrolysis. The bacterial strain is highly antagonistic to rice ShB and blast pathogens (34). The bacterium (*B. subtilis* strain AUBS1) also produces phenylalanine ammonia-lyase (PAL), peroxidase (PO) and certain pathogenesis-related proteins (PR) in rice leaves when applied against ShB disease. Application of bioagent also resulted in accumulation of thaumatin-like proteins, glucanases and chitinases

(42). Increased antagonistic abilities of *B. subtilis* (BS-916) were reported against *R. solani*, when the bioagent was implanted with N^+ at $150 \times 2.6 \times 10^{13}$ to $250 \times 2.6 \times 10^{13} N^+ / cm^2$. An increase in inhibition zone against ShB pathogen was noticed with the mutants to an extent of 4.3 to 31% under *in vitro* conditions. The control effect of the mutants is estimated to be 3.2 to 19% over that of BS-916 (64).

The efficacy of *Bacillus* spp against rice ShB disease is dependent on the antagonist population threshold in the soil. For effective suppression the population levels of the antagonist should be higher than 1×10^6 cfu/g during early infection of *R. solani* within 6-7 days (65). Mode of application of the bacterial bioagent and the type of formulation also affects its efficacy under greenhouse and field conditions. Floating pellet and water-soluble granule formulations of *B. megaterium* were found effective against rice ShB disease. Of these, foliar spraying of the bioagent was more effective than the floating pellet formulation in reducing the percent ShB affected tillers (46). In a separate study, it was reported that the floating pellet formulation of *B. megaterium* consisting of hydrogenated vegetable oil, lactose, microcrystalline cellulose, and a disintegrant, cross-linked sodium carboxy-methyl-cellulose showed promising result in suppression of rice ShB lesions in greenhouse experiment (123). The effervescent, fast-disintegrating granules, containing endospores of *B. megaterium* when either broadcasted or sprayed, reduced ShB infection in rice under greenhouse conditions. Further, the bacteria remained viable in effervescent granular form (10^9 cfu/g) even after one year of storage at room temperature. Even the number of viable and virulent bacteria after applying into water and spraying on rice seedlings in greenhouse were also satisfactory (10^9 and 10^6 cfu/g respectively) (124).

Bacillus spp exhibited synergistic effect when used in conjunction with other bio-pesticides. When used along with fungal bioagent such as *T. viride*, *B. subtilis* resulted in ShB disease reduction effectively in pot culture studies (25). When the bacterial antagonist was applied in combination with the other fungal bioagent *Gliocladium virens* effectively controlled ShB disease reduction (73%) at lower doses both the bioagents (2.5g/kg of *G. virens* and 10^8 cells/ml of *B. subtilis*) (104). It was also reported that the fermented product of *Bacillus* strain Drt-11 when used in combination with commercial biofungicide Jingga-meisu WP (20%) yielded significantly higher efficacies in rice ShB control than their individual applications (17).

Other bacteria showing antagonistic activity against ShB pathogen include *Streptomyces* spp and *Serratia marcescens*. Antifungal metabolites of *Streptomyces* spp (PM5, SPM5C-1 and SPM5C-2) were highly effective against the mycelial growth of rice ShB and blast pathogens under *in vitro* conditions. A complete inhibition was obtained at concentrations of 25, 50, 75 and 100 μ g/ml. Greenhouse studies indicated that spraying of SPM5C-2 @500 μ g/ml on rice significantly decreased ShB and blast disease development by 82 and 76% respectively (88). The antifungal activity of *Serratia marcescens* was reported. Culture filtrates of the bioagent showed enhanced biocontrol activity when combined with low concentrations of fungicides like flutolanil, penycuron and validamycin in terms of reducing sclerotial viability of ShB pathogen (110).

Integrated disease management. Integrated disease management (IDM) of rice ShB is gaining momentum and encompasses all the available control methods with each method compensating the deficiencies of others. Among the available IDM practices, combined use of chemical, cultural, biological and host plant resistance is a common phenomenon. However, host

plant resistance to ShB range only from very susceptible to moderately susceptible levels in rice (33), thus chemical management has become a necessary component for an effective IDM.

Combined applications of bioagent with chemical fungicides are an important IDM package against ShB. The use of fungal bioagents in controlling rice ShB in an IDM is gaining importance. Among the fungal bioagents, *Trichoderma* spp are important biocontrol agents that are effective against major soil borne diseases. Application of *T. harzianum* with soil organic amendements such as FYM, wheat straw, dhaincha (*Sesbania aculeata*), saw dust and neem cake worked effectively in managing rice ShB and also in increasing grain yields (54). Combined field applications of *T. viride* (5kg) and validmycin (2L)/ha was found to be effective in controlling ShB and sheath rot diseases of rice besides enhancing crop yield (23). Spray application of the spore suspension of *T. viride* (Tv3235) along with carbendazim (0.1%) and soil applications of FYM (1%) + saw dust (1%) showed maximum reduction in ShB severity, percent disease incidence and significant increase in grain yields over control (114).

Trichoderma spp were found to be compatible with majority of fungicides used in ShB management. *In vitro* studies revealed that fungal bioagents, *T. harzianum* and *G. virens*, are compatible with captan and are effective against ShB pathogen in rice. Integrated field evaluation proved that *Azolla pinnata* at 5t/ha as green manure along with FYM at 2.5 t/ha was highly effective in reducing the ShB disease incidence (14.63%) and increasing winter rice yield (40.29q/ha). FYM alone and *Sesbania aculeata* + FYM are the next best treatments. The interactive effects of seed/root dip treatment and amendements showed the best results in disease reduction and in yield increase (31). The bioagent *T. harzianum* was highly compatible with Hinosan (edifenphos) at 0.05% concentration. Field studies indicated that the bioagent was effective when combined with Contaf (hexaconazole), Hinosan, Rhizolex (tolelofoxmethyl), and

Validacin (validamycin). Hinosan is suggested as the best fungicide for combined application with *T. harzianum* due to its compatibility (5).

Plant growth promoting rhizobacteria (PGPR) offer a promising means of controlling plant diseases besides contributing to the plant resistance, growth and yield in rice (78). Of different PGPR, *Pseudomonads* and *Bacillus* spp were found to be very effective as a supplement in IDM. Greenhouse and field studies against rice ShB pathogen with different bacterial bioagents isolated from farmyard manure, rice seed, rice phyllosphere, and rice rhizosphere proved that three bacteria, PF-9 (*Pseudomonas fluorescens*), B-44 (*Bacillus* sp), and Chb-1 (chitinolytic bacterium) are compatible with carbendazim (Bavistin) at 500 and 1000 ppm concentrations. Among the three bioagents, PF-9 was most effective in reducing disease severity either alone or in combination with one spray of 0.1% Bavistin, followed by combination of PF-9 and B-44 (60). The bacterial bioagent, *B. subtilis* (Bs-916) when applied along with jinggangmycin was found to colonize the root system effectively. Further, the population density of BS-916 was maintained in its presence without any decline (19). In a separate study, it was found that the ShB disease was effectively controlled when jinggangmycin was mixed and sprayed with a growth regulator (Yi-Sui-Su) at the booting stage. A synergistic effect of the combination was noticed both in terms of reduction in disease severity as well as increase in growth and yield (126).

Other IDM packages that were found effective against ShB are combined use of botanicals, fungicides and organic amendments. It was reported that Achook (azadirachtin), a neem based chemical performed better with a ShB disease incidence of 65% compared to control (83%) (38). ShB disease development was found to be less coupled with more number of filled grains per

panicle, 1000-gram weight, straw and grain yields in pot culture studies with combined doses of ash, bleaching powder, poultry manure and Bavistin over control (8).

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CHAPTER 2

SCREENING OF PLANT GROWTH-PROMOTING RHIZOBACTERIA (PGPR) FOR SUPPRESSION OF *RHIZOCTONIA SOLANI* AND ENHANCEMENT OF RICE SEEDLING VIGOR

ABSTRACT

Sheath blight (ShB) of rice, caused by *Rhizoctonia solani* is an economically significant disease throughout the world. Management of ShB using plant growth-promoting rhizobacteria (PGPR) is an ecologically-friendly and viable alternative to current use of chemical fungicides. To select efficacious PGPR strains, approximately 70 isolates were screened for their antagonistic activity on vegetative growth and sclerotial germination of *R. solani* using dual *in-vitro* antibiosis assays. The strains were also screened for enhancement of rice seedling emergence under *in vitro* conditions. Ten PGPR strains were screened in each assay. In dual culture assays, mycelial plug of actively growing culture of *R. solani* was grown in Petri dishes containing 10% TSA and the PGPR strain was streak inoculated at 2 CM away on opposite sides. Plates were incubated at 25⁰ C for 5 days and observations on mycelial inhibition of *R. solani* by PGPR were taken. Surface sterilized sclerotia of *R. solani* were treated with liquid PGPR inoculum for 24 h and later plated onto Petri dishes containing PDA. There were five replications for each PGPR strain. Sclerotial germination and growth inhibition were observed after incubating for 5 days at 25⁰ C. To study the effect of PGPR strains on rice seedling vigor, surface sterilized rice seeds of Cv. Cocodrie in bacterial suspensions at a concentration of 4×10^8 cfu/ml for 24 h. The seeds were later dried and

incubated for 7 days. There were 3 replications and 25 seeds for each replication. *In vitro* antibiosis assays showed that the majority of the strains significantly suppressed the mycelial growth of *R. solani* compared to the control. Similarly, four out of 70 strains tested significantly suppressed sclerotial germination when the sclerotia were treated with PGPR suspensions. Majority of the strains significantly increased seedling vigor when tested as seed treatments under *in vitro* conditions. The strains *Bacillus amyloliquefaciens* AP 219, *B. subtilis* strains MBI 600 and AP 52 performed the best in these assays.

INTRODUCTION

A number of fungi, bacteria, virus, nematode and mycoplasma-like organisms cause disease on rice plants. Among the fungal diseases, sheath blight (ShB) caused by *Rhizoctonia solani* reduces yields up to 50% under optimum growing conditions (22) and is considered a serious constraint for rice production. The pathogen survives in the form of the dormant mycelia in previous crop stubble, as sclerotia in the soil for several years and on weeds (13, 14). Pathogen survival is mainly through sclerotia in rice fields. These sclerotia are irregularly shaped, brown to black colored, and are able to survive in soil for several years. The succeeding transplanting crop will be infected when these sclerotia float in the water and accumulate around plant bases under conditions of crop submergence. Infectivity of sclerotia is positively correlated to their size (16). Initial infections occur on sheaths of rice seedlings near the water line and subsequently the ShB lesions spreads vertically. Under favorable conditions, these lesions coalesce and in some cases, lodging of seedlings occur especially in taller cultivars.

Rice cultivars' resistance to ShB ranges from very susceptible to moderately susceptible (6). Currently, ShB is managed through systemic and non-systemic fungicides (19). Management

strategies mainly aimed at prevention of outbreak or epidemics through the use of both host plant resistance and chemical pesticides. The persistent, injudicious use of chemicals has toxic effects on non-target organisms and can cause undesirable changes in the environment. Many of these chemicals are too expensive for use by the farmers of Asia, where 90% of the world's rice is grown. Large-scale and long-term use of resistant cultivars is likely to result in shifts in the virulence characteristics of pathogens, culminating in resistance breakdown. Research during the previous two decades indicates another potential option for rice disease management, which is the use of plant growth-promoting rhizobacteria (PGPR). Use of PGPR assumes special significance as it is an ecologically-friendly and cost effective strategy which can be used in integration with other techniques for a greater level of protection with sustained rice yields.

Due to pesticide resistance, environmental pollution, and escalated crop protection costs, research on alternative disease management strategies is increasing. Research on PGPR for use on rice pathogens started in the 1980s. Research is still concentrated on the identification, evaluation and formulation of potential PGPR strains for deployment. For industry, meanwhile, ShB is economically important. It is one of the few crop diseases that justify the development of single-target fungicides. However, chemical fungicides present hazards to human health and the environment, and farmers in Asia are already rejecting them in favor of more sustainable approaches. But the Asian market is open to a range of chemicals, some of them so hazardous that their sales are restricted by several governments.

When fungicides are used intensively, they place enormous selection pressure on ShB, and the pathogen can develop resistance. Farmers are therefore left with the choice of using fungicides in moderation, which leaves the crop vulnerable to ShB, or beginning a cycle of heavier dosages of chemicals. Neither option is sustainable. Not only is chemical protection too

expensive but, even in moderation, there are indirect costs from the use of fungicides to the health of the farming family and the surrounding ecosystem, which put great strain on the grower's limited resources. As these problems have become more widely recognized, the international agriculture research institutions have responded by shifting their focus to biological agents.

One strategy for biological control of ShB is the use of PGPR. In previous studies, some of the PGPR provided significant suppression of *R. solani* and increased seedling growth when compared to other fungal antagonists. Rice plants and ecosystem are the natural habitat of many bacteria and rice ecosystem is rich in bacteria that are beneficial in pathogen control and in plant growth promotion (16). The populations of these bacteria however depend on the seed source, seed health and also on sowing of germinated or pre-germinated seed (4). In tropical rice fields, some bacteria are considered responsible for fungistatic of *R. solani* sclerotia, as evidenced by the finding that more than 80% of the non viable sclerotia were colonized by bacteria (S. Z. Yin and T. W. Mew, *unpublished data*). Of these, gram positive bacilli such as *B. subtilis*, *B. laterosporus* and *B. pumilus* were prevalent in rice ecosystems with potential antagonistic activity on rice pathogens besides plant growth promotion (23). Inoculation of rice seeds with bacteria can bring about positive changes in seedling growth and yield increase besides controlling plant pathogens (3). Seed bacterization with *B. vallismortis* EXTN-1 resulted in seedling growth promotion and suppression of sheath blight, blast and brown spot diseases (21). *Bacillus* spp. produces endospores that withstand desiccation, heat, oxidizing agents, and UV radiations (10). Earlier reports indicated that *Bacillus* spp. exhibited effective antagonism on ShB pathogen (26). They also produce certain antibiotics (5) and enzymes such as phenylalanine

ammonia- lyase (PAL), peroxidases, and pathogenesis-related proteins (PR) in rice leaves when applied against ShB disease (9).

To date, there are no published reports of the effect of PGPR on germination of sclerotia of *R. solani*. Because sclerotial germination is a key part of disease occurrence and rapid disease spread, any potential inhibition of sclerotial germination by PGPR would be helpful in disease management. Hence, the aim of the present study was to screen several PGPR strains with known plant growth-promoting and antagonistic activities in other crops and pathosystems for their antagonism to mycelial growth, and sclerotial germination of *R. solani*, as well as their influence on rice seedling vigor under *in vitro* conditions.

MATERIALS AND METHODS

Source of *Rhizoctonia solani*. A multinucleate and virulent isolate of *R. solani* anastomosis group AG-1 IA was obtained from the culture collection of Dr. D. E. Groth, Rice Research Station, LSU AgCenter, Crowley, Louisiana, USA. The isolate was originally isolated from ShB infected rice seedlings. The culture was maintained on potato dextrose agar (PDA) or on rye kernels for further use.

Source of PGPR strains. Approximately 70 PGPR strains were obtained from Dr. J. W. Kloepper, Department of Entomology and Plant Pathology, Auburn University, AL, USA., and used in this study. The selected strains possessed one or several of the following characteristics: (i) *in-vitro* antibiosis against various fungal pathogens, (ii) promotion of rhizobial root nodulation, (iii) enhancement of root and shoot growth of various crops and vegetables, and (iv) capacity to produce plant growth regulators. Purified and identified strains were grown for 48 h at 25⁰ C in 20 ml sterile tryptic soy broth (TSB) (Difco, Detroit, Michigan, USA) on a

reciprocating shaker (80 rpm). Bacteria were pelleted by centrifugation for 20 min at 10,000 x g. Bacterial cells were then washed (twice) in 0.1 M phosphate buffer (PB) (pH 6.8), resuspended in TSB amended with 20% sterile glycerol, and frozen in vials at -80⁰ C for long term storage. In each screening assay a new vial of PGPR was used.

Source of rice cultivar. A high-yielding, very early maturing long-grain cultivar of Cocodrie developed at Rice Research Station, LSU AgCenter, Crowley, Louisiana, USA was used throughout this study. The seeds were stored at 4⁰ C prior to use.

Influence of PGPR for suppression of vegetative growth of *Rhizoctonia solani*. A series of bio-assays were conducted to screen the efficacy of PGPR strains to inhibit the vegetative growth of *R. solani*. In each bio-assay, there were 10 PGPR strains and a control treatment. Each treatment was replicated five times. For testing PGPR strains, strains were retrieved from -80⁰ C freezer, thawed and streaked onto TSA and checked for purity after incubation for 24 h at 30⁰ C. A screening assay was conducted on 10% TSA plates by adopting a dual culture plate technique (7) against *R. solani*. Plugs of mycelium (5 mm diameter) were cut from the edge of an actively growing fungal colony on PDA with a No. 2 cork borer, and one plug was placed in the center of each TSA plate (100 x 15 mm). Two parallel 3.5 cm long streaks of bacteria were then made 2 CM apart on opposite sides of the plug. The pathogen not inoculated with the selective PGPR isolate served as control. The plates were incubated at 25⁰ C for 5 days in the dark. Five days after incubation, the inhibition of the mycelial growth of the pathogen was measured by using the formula:

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

where I = inhibition of mycelial growth of pathogen (%), C = growth of pathogen in the control plate (mm) and T = growth of pathogen in plates challenged with PGPR (mm). The width of the inhibition zone between PGPR and pathogen was measured after 7 days.

Influence of PGPR on the germination of sclerotia of *R. solani*. The effect of PGPR on sclerotial germination of *R. solani* was assayed by adopting the following procedure (11). For this assay, sclerotia of *R. solani* were produced on PDA at 25⁰C by incubating the inoculated plates for 10 days. Uniform sclerotia were collected from PDA plates and surface sterilized in 2.5% sodium hypochlorite solution for 2 min. In this assay, the same 70 PGPR strains used above were used. Seven screening assays were conducted. In each assay, there were 10 PGPR strains and an uninoculated control. As explained above, fresh culture of each PGPR strain was retrieved from -80⁰ C and streaked on TSA plates. A loopful of 24h old culture was inoculated into 250 ml flasks containing 50 ml of 10% TSB and incubated for 24h on a rotary shaker at 175 rpm at room temperature (26±2⁰ C). Five surface sterilized sclerotia were placed in each flask and incubated for another 24h on a rotary shaker under similar conditions as above. The sclerotia were later gently removed with sterile forceps and placed onto PDA plates and incubated for 5 days at 25⁰ C in the dark. Germination rate of the sclerotia and mycelial growth from germinated sclerotia were measured. Sclerotia incubated in sterile TSB without PGPR served as control. There were five replications for each PGPR strain and control in each assay. The % inhibition of mycelial growth from sclerotia was calculated using the following formula:

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

where I = inhibition of mycelial growth of pathogen (%), C = mycelial growth of sclerotia not challenged with PGPR (mm) and T = mycelial growth from sclerotia challenged with PGPR (mm).

Influence of PGPR on rice seedlings vigor index. Rice seeds (cv. Cocodrie) surface sterilized with 1% sodium hypochlorite solution for 2 min were used in seed germination assay. Surface sterilized seeds were treated with individual PGPR strains. For seed treatment, PGPR were produced in 10% TSB for 24 h on a rotary shaker at 26⁰ C and a concentration of 4 x 10⁸ cfu ml⁻¹ was used. Seeds were soaked in PGPR suspension for 24 h and germination of PGPR treated seed was tested using standard Roll Towel method (8). Seeds were later blot dried, and incubated in growth chamber for 7 days. There were 7 assays, and in each assay there were 10 PGPR strains and a control. There were three replications in each treatment, with 25 seeds per replication. Seeds soaked in sterile distilled water served as control. Percent germination of seeds, root and shoot lengths of seedlings were measured after 7 days and the seedling vigor index was calculated (1) as follows:

$$\text{Vigor index} = \% \text{ Germination} \times \text{Seedling growth (shoot length} + \text{root length)}$$

Statistical Analysis. The data were analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) and the treatment means were differentiated by a least significant difference (LSD) at P=0.05 using PROC- GLM.

RESULTS

Influence of PGPR on suppression of vegetative growth of *R. solani*. Of the 70 PGPR strains screened, the majority significantly reduced the mycelial growth of the ShB pathogen with percent inhibition ranging from 0 to 83% (Tables 1 through 7). Ten strains exhibited 70% inhibition or more. These superior strains include *Bacillus subtilis* strains MBI 600 and AP 52; *B. amyloliquefaciens* strains AP 219, AP 136, AP 188, and AP 295; *B. mycoides* strains ABU 1240, ABU 1627 and ABU 3586; and one strain of *B. simplex* ABU 3296. Of these superior

strains, the highest per cent inhibition was obtained with *B. subtilis* MBI 600 (Fig. 1.) The inhibition zones for these PGPR strains ranged from 0 to 5 mm.

Influence of PGPR on the germination of sclerotia of *Rhizoctonia solani*. Sclerotial germination of *R. solani* was completely inhibited by 4 of the 70 PGPR strains; MBI 600 (Fig. 2) (Table 2), AP 219, AP 52 (Table 1), and *B. simplex* strain ABU 1053 (Table 5). These strains also exhibited significant antagonism on mycelial growth of *R. solani*. Reduction in hyphal growth from sclerotia for other PGPR strains ranged from 6.7% to 42.7%.

Influence of PGPR on ice seedlings vigor index. The majority of the screened PGPR strains when applied to rice seeds were found to enhance germination, seedling length and thereby the vigor as observed on 7-day-old seedlings (Table 1 through Table 7). Highly effective strains in promoting seedling vigor include MBI 600 (Fig. 4), AP 219, AP 52, AP 295, ABU 1240, ABU 1627, ABU 3586, ABU 3296, and *Bacillus simplex* strain ABU 3099 with high seedling vigor (> 8,000) compared to control seedlings with seedling vigor in the range of 4867 to 5193 (Table 1 through Table 7). These superior strains were also found effective in reducing mycelial growth of ShB pathogen except for the strain ABU 3099 that showed moderate inhibition. Highest seedling vigor was obtained with PGPR strains AP 295 (13,600), followed by MBI 600 (13,192) with significant differences between them.

DISCUSSION

Studies on the effect of PGPR in inhibiting vegetative growth and sclerotial germination of *R. solani* are important in identifying potential strains for ShB management. In our present study, two strains of *B. subtilis* (MBI 600 and AP 52) and one strain of *B. amyloliquefaciens* (AP 219) were found effective in suppressing the vegetative growth of the mycelium (73 to 83%), inhibits

the sclerotial germination (100%) of *R. solani* and increased rice seedling vigor. PGPR offers a promising means of controlling plant diseases, besides contributing to resistance, growth and yield in rice (17). Epiphytic and endophytic bacterial strains isolated from healthy rice seed have been found to inhibit the mycelial growth of ShB pathogen (27). PGPR strains are known to produce siderophores, volatile metabolites, extra cellular secretions and antibiotics that are inhibitory to *R. solani*. Certain PGPR isolates also produce β 1, 3-glucanase, salicylic acid and HCN in inhibiting the mycelial growth of *R. solani* (18). Reduction in germination and lysis of *R. solani* sclerotia by PGPR have also been reported (12). Pre-treating the sclerotia with bacterial cell suspensions for 1 min to 4 weeks resulted in inactivation (20). Suppression of rice ShB pathogen, *R. solani* by non-fluorescent PGPR like *Bacillus* spp was previously attributed to the production of chitinase (15) and other antifungal metabolites. A large number of bacterial strains were found to possess the ability to protect rice plants from ShB disease (25) and these were identified through dual plate assays.

Seed treatment with bacterial antagonists also resulted in increased shoot and root lengths of seedlings (24). Use of diazotrophic bacteria in rice to promote growth and yields in rice is a common practice. Seed treatment with diazotrophs resulted in growth responses such as seedling emergence, radicle elongation, plumule length, cumulative leaf and root areas, grain and straw yields. The growth promotion in rice by diazotrophs is attributed to production of indole -3-acetic acid (2).

Effective inhibition of mycelial growth and sclerotial germination are pre-requisites for a candidate PGPR strain to be used for ShB management at field level. Since, sclerotial germination is a crucial step for ShB incidence, any strain that inhibits this process can be exploited for successful management of ShB. In addition, the selected strain should be able to

enhance seedling growth and yields. Further studies were planned to determine the efficacy of the superior strains identified in this screening bio-assays for management of ShB under greenhouse and field conditions. The use of PGPR's for management of ShB at field level at multiple locations can be explored. Understanding the mechanism of disease pathogen suppression is essential for the successful deployment of PGPR strains as a disease management strategy. Rice disease suppression by PGPR is governed by a multitude of factors. The success of a PGPR strain depends largely on the ability of the introduced agent to establish itself in the new environment and maintain a threshold population on the planting material or in the rhizosphere. The influence of these factors varies with the type of PGPR strain and the nature of the pathogen targeted for control. Future studies are directed in understanding the mechanism of ShB suppression by selective PGPR strains.

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Table 1. Effect of PGPR strains on vegetative growth and germination of sclerotia of *Rhizoctonia solani* and seedling vigor of rice (Assay 1).

Strain	Identification	% Inhibition of mycelial growth	Inhibition zone (mm)	% Inhibition of Sclerotial germination	Seedling Vigor Index
AP3	<i>Bacillus safensis</i>	33.1 ^d	0 ^c	20 ^{cd}	5964 ^f
AP7	<i>Bacillus safensis</i>	55.6 ^c	2 ^{bc}	30.6 ^b	6404 ^{cde}
AP18	<i>Bacillus pumilus</i>	35.2 ^d	2 ^{bc}	17.3 ^d	5171 ^g
AP40	<i>Bacillus anthracis</i>	60.7 ^{bc}	4.6 ^a	16 ^d	6283 ^{def}
AP52	<i>Bacillus subtilis subsp. subtilis</i>	73.3 ^a	4.6 ^a	100 ^a	8124 ^b
AP136	<i>Bacillus amyloliquefaciens</i>	74.5 ^a	5 ^a	34.6 ^b	6246 ^{ef}
AP188	<i>Bacillus amyloliquefaciens</i>	69.4 ^{ab}	4 ^{ab}	36 ^b	6614 ^{cd}
AP209	<i>Bacillus subtilis subsp. subtilis</i>	63.5 ^{abc}	4 ^{ab}	28 ^{bc}	6686 ^c
AP217	<i>Bacillus macauensis</i>	0 ^e	0 ^c	17.3 ^d	5961 ^f
AP219	<i>Bacillus amyloliquefaciens</i>	74.5 ^a	4 ^{ab}	100 ^a	8864 ^a
Control	--	--	--	--	5193 ^g

Means followed by a common letter in the columns are not significantly different at $p \leq 0.05$.

¹Mycelial growth was recorded at 5 days after incubation and % inhibition was calculated over control.

²Width of inhibition zone between pathogen and PGPR was measured at 7 days after incubation.

³Sclerotial growth was recorded at 5 days after incubation.

⁴Seedling vigor index was calculated at 7 days after incubation.

Table 2. Effect of PGPR strains on vegetative growth and germination of sclerotia of *Rhizoctonia solani* and seedling vigor of rice (Assay 2).

Strain	Identification	% Inhibition of mycelial growth	Inhibition zone (mm)	% Inhibition of Sclerotial germination	Seedling Vigor Index
AP278	<i>Bacillus subtilis subsp. subtilis</i>	61.1 ^{bc}	5 ^a	22.6 ^{bcd}	8145 ^c
AP279	<i>Bacillus subtilis subsp. subtilis</i>	56.0 ^c	2 ^c	24 ^{bcd}	5390 ^f
AP280	<i>Bacillus safensis</i>	27.4 ^d	0 ^d	25.3 ^{bcd}	6456 ^d
AP281	<i>Bacillus safensis</i>	20 ^{de}	0 ^d	14.6 ^d	5610 ^f
AP282	<i>Lysinibacillus boronitolerans</i>	0 ^e	0 ^d	22.6 ^{bcd}	5561 ^f
AP283	<i>Bacillus safensis</i>	65.4 ^{abc}	3 ^{bc}	17.3 ^{cd}	7892 ^c
AP294	<i>Paenibacillus peoriae</i>	66.6 ^{abc}	4.3 ^{ab}	32 ^b	5985 ^e
AP295	<i>Bacillus amyloliquefaciens</i>	77.6 ^{ab}	4 ^{ab}	29.3 ^{bc}	13600 ^a
MBI 600	<i>Bacillus subtilis</i>	83.1 ^a	5 ^a	100 ^a	13192 ^b
AP302, 299	<i>Bacillus amyloliquefaciens</i>	50.1 ^c	1.3 ^{cd}	36 ^b	6644 ^d
Control	Control	--	--	--	5028 ^g

Means followed by a common letter in the columns are not significantly different at $p \leq 0.05$

¹Mycelial growth was recorded at 5 days after incubation and % inhibition was calculated over control

²Width of inhibition zone between pathogen and PGPR was measured at 7 days after incubation

³Sclerotial growth was recorded at 5 days after incubation

⁴Seedling vigor index was calculated at 7 days after incubation

Table 3. Effect of PGPR strains on vegetative growth and germination of sclerotia of *Rhizoctonia solani* and seedling vigor of rice (Assay 3).

Strain	Identification	% Inhibition of mycelial growth	Inhibition zone (mm)	% Inhibition of Sclerotial germination	Seedling Vigor Index
AP304	<i>Bacillus amyloliquefaciens</i>	50.5 ^{ab}	2.6 ^b	28 ^a	6693 ^{de}
AP305	<i>Bacillus amyloliquefaciens</i>	56.4 ^{ab}	4.3 ^a	20 ^{ab}	7160 ^c
ABU 29	<i>Bacillus simplex</i>	47.8 ^{ab}	1.6 ^{bc}	14.6 ^b	6960 ^{cd}
ABU 89B	<i>Bacillus simplex</i>	23.9 ^d	0 ^d	21.3 ^{ab}	6327 ^e
ABU 161	<i>Bacillus megaterium</i>	50.9 ^{ab}	1.6 ^{bc}	18.6 ^{ab}	7998 ^a
ABU 169	<i>Bacillus megaterium</i>	33.7 ^{cd}	0.6 ^{cd}	18.6 ^{ab}	5522 ^f
ABU 279	<i>Bacillus cereus</i>	44.3 ^{bc}	1.3 ^{bcd}	22.6 ^{ab}	6630 ^{de}
ABU 288	<i>Bacillus megaterium</i>	24.7 ^d	0 ^d	22.6 ^{ab}	4810 ^g
ABU 334	<i>Bacillus simplex</i>	56.4 ^{ab}	2 ^{bc}	25.3 ^a	5461 ^f
ABU 354	<i>Bacillus cereus</i>	59.2 ^a	4.3 ^a	18.6 ^{ab}	7583 ^b
Control	--	--	--	--	4959 ^g

Means followed by a common letter in the columns are not significantly different at $p \leq 0.05$

¹Mycelial growth was recorded at 5 days after incubation and % inhibition was calculated over control

²Width of inhibition zone between pathogen and PGPR was measured at 7 days after incubation

³Sclerotial growth was recorded at 5 days after incubation

⁴Seedling vigor index was calculated at 7 days after incubation

Table 4. Effect of PGPR strains on vegetative growth and germination of sclerotia of *Rhizoctonia solani* and seedling vigor of rice (Assay 4).

Strain	Identification	% Inhibition of mycelial growth	Inhibition zone (mm)	% Inhibition of Sclerotial germination	Seedling Vigor Index
ABU 361	<i>Bacillus simplex</i>	3.1 ^e	0 ^b	12 ^b	5355 ^{cd}
ABU 371	<i>Bacillus megaterium</i>	40.7 ^{ab}	0 ^b	18.6 ^{ab}	5462 ^c
ABU 402	<i>Bacillus weihenstephanensis</i>	15.6 ^{cde}	0.6 ^a	21.3 ^{ab}	5020 ^d
ABU 457	<i>Bacillus simplex</i>	40 ^{ab}	0.6 ^{ab}	24 ^a	6120 ^b
ABU 524	<i>Bacillus simplex</i>	43.9 ^a	0.6 ^{ab}	20 ^{ab}	6780 ^a
ABU 871	<i>Bacillus simplex</i>	9.4 ^{de}	0 ^b	10.6 ^b	5453 ^{cd}
ABU 882	<i>Bacillus megaterium</i>	25.1 ^{bcd}	0 ^b	17.3 ^{ab}	6128 ^b
ABU 890	<i>Bacillus simplex</i>	45.1 ^a	1.3 ^a	26.6 ^a	6987 ^a
ABU 891	<i>Bacillus simplex</i>	20 ^{cd}	0 ^b	26.6 ^a	6269 ^b
ABU 1025	<i>Bacillus simplex</i>	27.8 ^{bc}	0 ^b	18.6 ^{ab}	5137 ^{cd}
Control	--	--	--	--	5064 ^c

Means followed by a common letter in the columns are not significantly different at $p \leq 0.05$

¹Mycelial growth was recorded at 5 days after incubation and % inhibition was calculated over control

²Width of inhibition zone between pathogen and PGPR was measured at 7 days after incubation

³Sclerotial growth was recorded at 5 days after incubation

⁴Seedling vigor index was calculated at 7 days after incubation

Table 5. Effect of PGPR strains on vegetative growth and germination of sclerotia of *Rhizoctonia solani* and seedling vigor of rice (Assay 5).

Strain	Identification	% Inhibition of mycelial growth	Inhibition zone (mm)	% Inhibition of Sclerotial germination	Seedling Vigor Index
ABU 1053	<i>Bacillus simplex</i>	57.2 ^b	3 ^a	100 ^a	8218 ^b
ABU 1240	<i>Bacillus mycoides</i>	71.7 ^a	0 ^b	36 ^b	9224 ^a
ABU 1419A	<i>Bacillus simplex</i>	45.1 ^c	0 ^b	20 ^{de}	4962 ^e
ABU 1627	<i>Bacillus mycoides</i>	74.5 ^a	0 ^b	25.3 ^{cd}	9400 ^a
ABU 1645	<i>Bacillus simplex</i>	0 ^e	0 ^b	29.3 ^{bc}	5136 ^{de}
ABU 1687	<i>Bacillus simplex</i>	9.0 ^d	0 ^b	6.6 ^g	5805 ^{cd}
ABU 1930	<i>Bacillus simplex</i>	1.1 ^e	0 ^b	16 ^{ef}	5488 ^{cde}
ABU 1966	<i>Paenibacillus taichungensis</i>	9.8 ^d	0 ^b	10.6 ^{fg}	5220 ^{cde}
ABU 1970	<i>Bacillus simplex</i>	1.1 ^e	0 ^b	17.3 ^{def}	5822 ^c
ABU 2002	<i>Bacillus simplex</i>	2.7 ^e	0 ^b	16 ^{ef}	5454 ^{cde}
Control	--	--	--	--	4867 ^e

Means followed by a common letter in the columns are not significantly different at $p \leq 0.05$

¹Mycelial growth was recorded at 5 days after incubation and % inhibition was calculated over control

²Width of inhibition zone between pathogen and PGPR was measured at 7 days after incubation

³Sclerotial growth was recorded at 5 days after incubation

⁴Seedling vigor index was calculated at 7 days after incubation

Table 6. Effect of PGPR strains on vegetative growth and germination of sclerotia of *Rhizoctonia solani* and seedling vigor of rice (Assay 6).

Strain	Identification	% Inhibition of mycelial growth ¹	Inhibition zone ² (mm)	% Inhibition of Sclerotial germination ³	Seedling Vigor Index ⁴
ABU 2017	<i>Bacillus simplex</i>	64.7 ^a	4.6 ^a	24 ^{ab}	7047 ^a
ABU 2041A	<i>Bacillus simplex</i>	0 ^h	0 ^c	14.6 ^{cde}	5850 ^d
ABU 2099B	<i>Bacillus simplex</i>	18.0 ^d	0 ^c	28 ^{ab}	5823 ^d
ABU 2197	<i>Bacillus simplex</i>	6.6 ^{fg}	0 ^c	12 ^{de}	5060 ^e
ABU 2213	<i>Bacillus simplex</i>	2.3 ^{gh}	0 ^c	6.6 ^e	6398 ^b
ABU 2252	<i>Bacillus megaterium</i>	11.3 ^{ef}	0 ^c	20 ^{bcd}	6255 ^{bc}
ABU 2424	<i>Bacillus simplex</i>	3.1 ^{gh}	0 ^c	21.6 ^{cde}	5647 ^d
ABU 2429B	<i>Bacillus megaterium</i>	12.5 ^e	0 ^c	14.6 ^{cde}	6213 ^{bc}
ABU 2549	<i>Bacillus mycoides</i>	52.9 ^b	1 ^b	21.3 ^{abc}	5946 ^{cd}
ABU 2644	<i>Bacillus simplex</i>	23.5 ^c	0 ^c	29.3 ^a	5956 ^{cd}
Control	--	--	--	--	4904 ^e

Means followed by a common letter in the columns are not significantly different at $p \leq 0.05$

¹Mycelial growth was recorded at 5 days after incubation and % inhibition was calculated over control

²Width of inhibition zone between pathogen and PGPR was measured at 7 days after incubation

³Sclerotial growth was recorded at 5 days after incubation

⁴Seedling vigor index was calculated at 7 days after incubation

Table 7. Effect of PGPR strains on vegetative growth and germination of sclerotia of *Rhizoctonia solani* and seedling vigor of rice (Assay 7).

Strain	Identification	% Inhibition of mycelial growth	Inhibition zone (mm)	% Inhibition of Sclerotial germination	Seedling Vigor Index
ABU 2772	<i>Bacillus subtilis</i>	54.9 ^b	3 ^{bc}	40 ^a	6237 ^d
ABU 3099	<i>Bacillus simplex</i>	48.2 ^{cd}	0 ^d	26.6 ^{abc}	8398 ^b
ABU 3118	<i>Bacillus simplex</i>	7.8 ^e	0 ^d	9.3 ^c	4998 ^f
ABU 3128	<i>Bacillus simplex</i>	48.6 ^{cd}	0 ^d	25.3 ^{abc}	7595 ^c
ABU 3135	<i>Bacillus weihenstephanensis</i>	49.8 ^{bcd}	0 ^d	33.3 ^{ab}	5434 ^{ef}
ABU 3296	<i>Bacillus simplex</i>	74.5 ^a	4 ^a	28 ^{ab}	9788 ^a
ABU 3421A	<i>Bacillus vallismortis</i>	5.1 ^e	0 ^d	34.6 ^{ab}	5289 ^{ef}
ABU 3454	<i>Bacillus weihenstephanensis</i>	50.9 ^{bc}	2.6 ^c	32 ^{ab}	5753 ^{de}
ABU 3586	<i>Bacillus mycoides</i>	70.1 ^a	3.6 ^a	42.6 ^a	8301 ^{bc}
ABU 3819	<i>Bacillus aerophilus</i>	45.1 ^d	0 ^d	18.6 ^{bc}	5289 ^{ef}
Control	--	--	--	--	4925 ^f

Means followed by a common letter in the columns are not significantly different at $p \leq 0.05$

¹Mycelial growth was recorded at 5 days after incubation and % inhibition was calculated over control

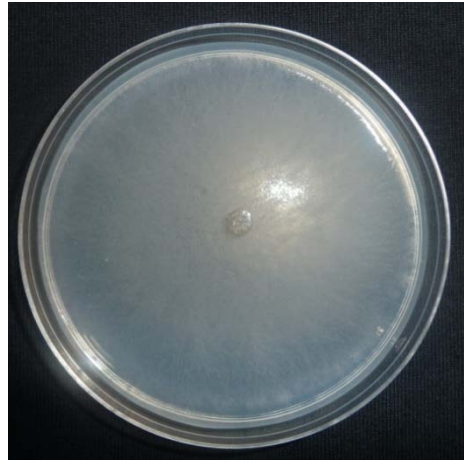
²Width of inhibition zone between pathogen and PGPR was measured at 7 days after incubation

³Sclerotial growth was recorded at 5 days after incubation

⁴Seedling vigor index was calculated at 7 days after incubation



Challenged with MBI 600



Control

Fig. 1. Inhibition of vegetative growth of *R. solani* by *Bacillus subtilis* MBI 600 in a dual plate assay.



Challenged with MBI 600



Control

Fig. 2. Inhibition of sclerotial germination of *R. solani* by *Bacillus subtilis* MBI 600 in a plate assay.

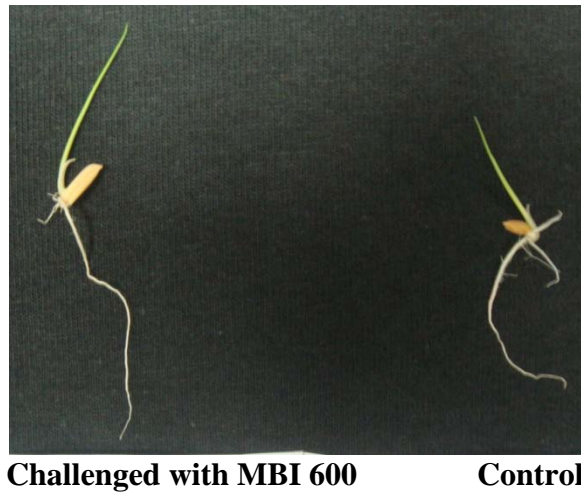


Fig. 3. Effect of seed bacterization with *Bacillus subtilis* MBI 600 on rice seedling vigor index.

CHAPTER 3

EVALUATION OF PLANT GROWTH-PROMOTING RHIZOBACTERIA FOR SUPPRESSION OF SHEATH BLIGHT OF RICE IN A DETACHED LEAF ASSAY

ABSTRACT

Sheath blight (ShB) of rice, caused by *Rhizoctonia solani*, is one of the most important rice diseases worldwide. The objective of this study was to optimize a disease assay on a detached rice leaf to screen selected plant growth-promoting rhizobacteria (PGPR) strains for suppression of ShB under controlled conditions. Sclerotia of *R. solani* were produced on PDA and designated as immature (< 5-day-old), mature (5-30-day-old), and aged (>30-day-old). Leaves of 60-day-old rice plants grown under greenhouse conditions were used to develop the disease assay. Leaf sections cut to of 8 cm in length were cut and placed in Petri dishes, inoculated with each of three different inoculum types of sclerotia, and incubated in a growth chamber. ShB lesions were assessed after 7 days. Immature sclerotia significantly produced longer lesions (5.8 cm) than did mature (4.9 cm) and aged sclerotia (5.0 cm). The efficiency of *R. solani* infection induced by immature sclerotia was significantly higher than that of mature and aged sclerotia. Approximately 70 PGPR strains were screened for suppression of ShB disease using the optimized assay by inoculating the leaves with immature sclerotia. The disease was quantified by the Relative Lesion Height (RLH) method. Among 70 strains, 31 significantly suppressed the RLH of ShB lesions compared to the control. Among these, *Bacillus subtilis* strain MBI 600 resulted in greatest suppression of ShB disease severity under the conditions tested.

INTRODUCTION

Sheath blight (ShB) of rice, caused by the *Rhizoctonia solani* Kühn (teleomorph: *Thanatephorus cucumeris* (A.B. Frank) Donk), is a major disease of rice, reducing both grain yield and quality. The pathogen is soil-borne and has a wide host range, often infecting legume crops grown in rotation with rice. Yield losses up to 50% have been reported with rice ShB. The disease is often severe in intense crop production systems especially when susceptible varieties are grown (13). In midsouth rice-producing areas of the USA, ShB is the most economically important disease (6, 13, 14). The pathogen survives in the form of sclerotia and mycelia in plant debris and on weeds in tropics (12). Strong sources of genetic resistance to ShB are not available. In general, all the rice cultivars are susceptible to ShB; however, the degree of susceptibility varies (22). In the United States, host plant resistance among cultivable varieties currently ranges from susceptible to moderately susceptible levels (5). Presently, the disease is being managed through the application of systemic fungicides and antibiotics to seed (8), soil (2), and foliage (3, 13). Use of fungicides in ShB management produces several concerns relating to environmental pollution, pathogen resistance, and escalated costs.

Use of PGPR in ShB management of rice is gaining popularity as an alternative to the chemical fungicides. Although ShB pathogen is soil-borne in nature, disease initiation occurs at the base of seedlings near water line, and the disease subsequently spreads through foliage (17). Therefore, the use of PGPR with good colonization potential in the rhizosphere and or phyllosphere is needed for successful control of ShB disease spread under field conditions. Identification of superior PGPR strains with high antagonistic potential to ShB pathogen and lesion spread on foliage is a vital step for devising effective biological control strategies at field

level. For this purpose, a rapid and reliable assay to screen the best performing PGPR strains under controlled conditions is essential.

Optimization of a ShB assay is a pre-requisite for initiation of PGPR research on rice disease management. Different screening methods for control of ShB were earlier reported using different inoculum sources of *R. solani* (1, 22, 16). The different inoculation procedures involved the use of *R. solani* on colonized tooth picks (18, 19, 27), agar plugs (4, 10), rice grain-hulls (11, 15, 26), mycelia fragments (20, 25), and sclerotia (22). Liquid cultured mycelia balls and mycelial suspensions were tested in another study (16). Sclerotia of different ages were also used to develop disease assays on rice seedlings (22). Among different aged sclerotia screened, lacing of immature sclerotia inside the leaf sheath with a few drops of sterile water induced discrete and uniform sized lesions under greenhouse conditions (22).

None of the previously published assays has been used for screening of PGPR strains for suppression of ShB lesion disease under controlled conditions. Therefore, the objectives of the present study were i) to develop an optimized ShB disease assay on a detached leaf assay under laboratory conditions, and ii) to screen selective PGPR strains with known activities on an optimized detached leaf assay for selection of best performing strains for control of ShB disease in rice seedlings. The information thus generated will be useful in selecting PGPR strains that potentially reduce spread of the ShB lesions on the rice plant.

MATERIALS AND METHODS

Source of pathogen and production of sclerotia of *Rhizoctonia solani*. A multinucleate and virulent isolate of *R. solani* anastomosis group AG-1 IA was obtained from the culture collection of Dr. D. E. Groth, Rice Research Station, LSU AgCenter, Crowley, Louisiana, USA.

The isolate was originally isolated from ShB infected rice seedlings. The culture was maintained on potato dextrose agar (PDA) or on rye kernels for further use. For production of sclerotia, *R. solani* was grown on PDA at $28\pm 1^{\circ}\text{C}$ under dark. The sclerotia were harvested at different time intervals and categorized according to their age as follows: immature (<5-day-old), mature (5-30 day-old and aged (>30-days-old). The selected sclerotia were stored at 4°C prior to use.

Source of PGPR strains. Approximately 70 PGPR strains were obtained from the Phytobacteriology Laboratory strain collection, Department of Entomology and Plant Pathology, Auburn University, AL, USA. The selected strains possessed one or several of the following characteristics: (i) *in vitro* antibiosis against various fungal pathogens, (ii) promotion of root growth on several crops, (iii) enhancement of root and shoot growth of various crops and vegetables, and (iv) capacity to produce plant growth regulators. Purified and identified strains were grown for 48 h at 25°C in 20 ml sterile tryptic soy broth (TSB) (Difco, Detroit, Michigan, USA) on a reciprocating shaker (80 rpm). Bacteria were pelleted by centrifugation for 20 min at $10,000 \times g$. Bacterial cells were then washed (twice) in 0.1 M phosphate buffer (PB) (pH 6.8), resuspended in TSB amended with 20% sterile glycerol, and frozen in vials at -80°C for long term storage. In each screening assay a new vial of PGPR was used.

Production of rice seedlings. Seeds of a high-yielding, very early maturing long-grain rice, CV. Cocodrie, developed at Rice Research Station, LSU AgCenter, Crowley, Louisiana, USA, were used. Rice seedlings were produced in plastic pots containing field soil amended with Osmocote fertilizer under greenhouse conditions. Pots were initially filled with tap water and the soil was soaked completely for 72 h. Later the soil was agitated manually to break the aggregates, and excess water was drained. Rice seedlings were produced by sowing two seeds per pot and placed on a bench in the greenhouse. Seedlings were under submerged conditions

from 4th leaf stage. The pots were maintained at a temperature of 26 ± 2 C, and RH of 90 for 60 days.

Optimization of detached leaf assay for ShB disease. Leaves at second position from the base of the culm from 60-day-old rice seedlings grown as above in the greenhouse were detached and brought to the laboratory in an ice box for ShB disease optimization (7). Leaves were cut into uniform sizes of 8 cm long. These leaves were surface sterilized with 1% sodium hypochlorite solution for 2 min. An individual leaf was then placed in Petri dishes of 14 CM diameter containing moistened filter papers. The leaves were supported by clean glass slides at the ends to prevent the leaves from rolling inwards. Immature, mature and aged sclerotia produced on a PDA as above were used as a source of pathogen inoculum. To assess, immature, mature and aged sclerotia were inoculated individually by placing one sclerotium at the center of a leaf (22). The Petri dishes with leaves were later placed in plastic trays lined with moistened filter paper. The trays were incubated in a growth chamber at 25 ± 1^0 C and 16 h light. There were five replications for each age of sclerotia, one petri dish per replication. Observations of lesions on the leaves were made, and the size of the lesions was measured at 7 days after incubation.

Evaluation of select PGPR strains for suppression of ShB in a detached leaf assay. Seventy PGPR strains as described in Tables 2 through 8 were screened for their efficacy in the suppression of ShB symptoms in a detached leaf assay (7). In each assay, there were 10 PGPR strains and a control treatment. Each treatment was replicated five times. For testing PGPR strains, strains were retrieved from -80^0 C freezer, thawed, and streaked onto TSA and checked for purity after incubation for 24 h at 30^0 C. Cell suspensions of PGPR were prepared by growing the strains for 48 h at 25^0 C in TSA, harvesting in sterile distilled water, and adjusting the final concentrations at 4×10^8 cfu ml⁻¹. Leaves from 60-day-old seedlings produced as above

in the GH were cut and brought to the laboratory in an ice box and surface sterilized as described above. They were then cut into uniform sizes of 8 cm and placed in sterilized glass Petri dishes of 14 CM diameter containing moistened filter paper. There was one leaf piece per Petri dish per replicate of the PGPR strain. In each Petri dish, surface sterilized glass slides were placed on the edges of these leaf pieces to prevent rolling inwards. Each PGPR strain was sprayed onto the surface of leaf pieces in the Petri dish. One immature sclerotium of *R. solani* produced on PDA was placed at the center of the leaf piece. Leaves sprayed with sterile distilled water with inoculated sclerotium served as control treatment in each assay. The Petri dishes with leaves were later placed in plastic trays lined with moistened filter paper. The trays were incubated in a growth chamber at $25\pm 1^{\circ}$ C and 16 h light. At 7 days after incubation, leaves were rated for ShB disease lesions. The lesion length around the sclerotium was measured and ShB severity was rated by the Relative Lesion Height (RLH) method (21) with the following formula:

$$\% \text{ RLH} = 100 \times \text{Total height of lesions} / \text{Total leaf height}$$

Statistical analysis. The data were analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) and the treatment means were differentiated by a least significant difference (LSD) at $P=0.05$ using PROC- GLM.

RESULTS

Optimization of detached leaf assay for ShB disease. Inoculation of detached leaves with different ages of sclerotia provided ShB lesions at various degrees. Lesion lengths with immature sclerotia ranged from 55-60 mm. Lesion length was highest with immature sclerotia (57.5 mm) (Fig. 1) and was significantly superior over that of mature (48.5 mm) and aged sclerotia (49.5 mm). There were no significant differences in lesion lengths provided by mature and aged

sclerotia (Table 1). As the leaf lesions aged, new sclerotia developed on the mature lesions (Fig. 2).

Evaluation of PGPR for suppression of ShB disease in a detached leaf assay. Of the 70 PGPR tested, only 31 PGPR strains have significantly reduced the ShB lesions when compared to the control (Tables 2 through 8). The disease severity in these significant PGPR strains ranged from 2.9% to 93.3%. Among the PGPR strains tested, maximum inhibition of lesion development was obtained with *B. subtilis* MBI 600 with 2.9% of disease severity (Fig. 3). The next best reduction of ShB was noticed with *B. subtilis subsp. subtilis* strains AP 209 and AP 52 (with 32.1% and 39.58% disease, respectively), and one strain of *B. amyloliquefaciens* AP 219 with 39.2% disease severity.

DISCUSSION

We have successfully developed a rapid and reliable ShB disease assay on detached rice leaves for screening different PGPR under controlled conditions. Previously, several researchers worked on different inoculation methods for evaluating rice germplasm for ShB resistance (24). Improved inoculation techniques of ShB were earlier evaluated to devise simple, less time consuming and highly reproducible assays on rice seedlings under greenhouse conditions (22).

The detached leaf inoculation technique was earlier attempted for determining the morphological and pathological variability in rice isolates of *R. solani* and molecular analysis of their genetic variability (7). The assay was found to be useful in determining the host specific toxin production by *R. solani* in rice (23). Use of detached leaves for assays is less time consuming compared to whole-plant assays under greenhouse conditions. Our research results showed that inoculation of rice leaves with different aged sclerotia resulted in different degrees

of ShB symptoms. However, inoculation of rice leaves with immature sclerotium, resulted in uniform symptoms with a lesion size that was significantly superior to lesions induced by other sclerotia. Earlier studies on use of different aged sclerotia indicated that youngest sclerotia produced largest ShB lesions at 96 h after inoculation on rice seedlings under greenhouse conditions (22). Sclerotia of different ages and sizes differ widely with respect to germination rate (22).

Evaluation of PGPR under laboratory conditions using detached leaf assay is the first step for identifying PGPR strains for disease management at the field level. In this study, the optimized assay allowed a consistent and reproducible inoculation of the sheath blight pathogen, resulting in an accurate measurement of disease severity. Because the disease assay uses rice plants at late tillering stage, it requires a longer duration of time. However, the disease assay may complement the seedling-based quick screening in determining superior PGPR strains against ShB disease. Since, the pathogen is soil-borne, and subsequent spread of the disease is through infection of the foliage (17). Therefore, foliar application of PGPR is essential for management of ShB under field conditions. PGPR, when applied to rice leaves, produce substances, such as phenylalanine ammonia-lyase, peroxidases, chitinases, glucanases, thaumatin-like proteins, and PR proteins, which may inhibit the severity of ShB disease (9). In our evaluation of PGPR, strains *Bacillus subtilis* MBI 600, *B. subtilis* subsp *subtilis* AP 209 and AP 52, and *B. amyloliquefaciens* AP 219 were highly effective in reducing ShB lesions on detached rice leaves.

Overall, strain MBI 600 significantly reduced ShB lesions on rice leaves and was the best strain compared to other strains tested. In our earlier studies (Chapter 2), MBI 600 showed significant reduction in mycelial growth of *R. solani*. Also the sclerotial germination of *R. solani* was completely inhibited by MBI 600 under *in vitro* conditions, and the strain significantly

improved seedling vigor. Strain MBI 600 was found to be the superior to the other tested strains and was selected for further studies. Further studies are needed on this strain to determine the growth promoting characteristics, its compatibility with commonly used fungicides, mode of action against *R. solani*, and suppression of ShB under greenhouse and field conditions.

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Table 1. Sheath blight lesion development on detached leaf of rice by different aged sclerotia of *Rhizoctonia solani* under controlled conditions

Age of sclerotia	Lesion length (mm)*	
	Range	Mean lesion length
Immature sclerotia (<5-day-old)	55-60	57.5 ^a
Mature sclerotia (5 - 30-day-old)	48-52	48.5 ^b
Aged sclerotia (>30-day-old)	46-50	49.5 ^b

Observations are the means of 5 replications

* Values are estimated at 7-days after inoculation

Means within a column followed by different letter(s) are significantly different ($P \leq 0.05$)

Table 2. Efficacy of PGPR strains on suppression of rice sheath blight in a detached leaf assay (Assay 1).

Strain	Identification	Sheath blight lesion spread¹
AP3	<i>Bacillus safensis</i>	100 ^a
AP7	<i>Bacillus safensis</i>	58.3 ^{bc}
AP18	<i>Bacillus pumilus</i>	100 ^a
AP40	<i>Bacillus anthracis</i>	87.5 ^a
AP52	<i>Bacillus subtilis subsp. subtilis</i>	39.5 ^d
AP136	<i>Bacillus amyloliquefaciens</i>	65.4 ^b
AP188	<i>Bacillus amyloliquefaciens</i>	45.8 ^{cd}
AP209	<i>Bacillus subtilis subsp. subtilis</i>	32.0 ^d
AP217	<i>Bacillus macauensis</i>	91.6 ^a
AP219	<i>Bacillus amyloliquefaciens</i>	39.1 ^d
Control	--	100 ^a

Means followed by a common letter within a column are not significantly different at $p \leq 0.05$

¹Sheath blight lesion spread was recorded at 5 days after incubation by Highest Relative Lesion Height method.

Table 3. Efficacy of PGPR strains on suppression of rice sheath blight in a detached leaf assay (Assay 2).

Strain	Identification	Sheath blight lesion spread¹
AP278	<i>Bacillus subtilis</i> subsp. <i>Subtilis</i>	64.5 ^{cd}
AP279	<i>Bacillus subtilis</i> subsp. <i>Subtilis</i>	41.6 ^e
AP280	<i>Bacillus safensis</i>	100 ^a
AP281	<i>Bacillus safensis</i>	90 ^a
AP282	<i>Lysinibacillus boronitolerans</i>	87.5 ^{ab}
AP283	<i>Bacillus safensis</i>	100 ^a
AP294	<i>Paenibacillus peoriae</i>	70.8 ^{bc}
AP295	<i>Bacillus amyloliquefaciens</i>	48.7 ^{de}
MBI 600	<i>Bacillus subtilis</i>	2.9 ^f
AP302, 299	<i>B. amyloliquefaciens</i>	62.5 ^{cd}
Control	--	100 ^a

Means followed by a common letter within a column are not significantly different at $p \leq 0.05$

¹Sheath blight lesion spread was recorded at 5 days after incubation by Highest Relative Lesion Height method.

Table 4. Efficacy of PGPR strains on suppression of rice sheath blight in a detached leaf assay (Assay 3).

Strain	Identification	Sheath blight lesion spread¹
AP304	<i>Bacillus amyloliquefaciens</i>	37.5 ^e
AP305	<i>Bacillus amyloliquefaciens</i>	56.2 ^{cd}
ABU 29	<i>Bacillus simplex</i>	100 ^a
ABU 89B	<i>Bacillus simplex</i>	95.8 ^a
ABU 161	<i>Bacillus megaterium</i>	58.3 ^c
ABU 169	<i>Bacillus megaterium</i>	100 ^a
ABU 279	<i>Bacillus cereus</i>	100 ^a
ABU 288	<i>Bacillus megaterium</i>	93.3 ^a
ABU 334	<i>Bacillus simplex</i>	50 ^d
ABU 354	<i>Bacillus cereus</i>	78.3 ^b
Control	--	100 ^a

Means followed by a common letter within a column are not significantly different at $p \leq 0.05$
¹Sheath blight lesion spread was recorded at 5 days after incubation by Highest Relative Lesion Height method.

Table 5. Efficacy of PGPR strains on suppression of rice sheath blight in a detached leaf assay (Assay 4).

Strain	Identification	Sheath blight lesion spread¹
ABU 361	<i>Bacillus simplex</i>	91.6 ^b
ABU 371	<i>Bacillus megaterium</i>	100 ^a
ABU 402	<i>Bacillus weihenstephanensis</i>	93.3 ^b
ABU 457	<i>Bacillus simplex</i>	100 ^a
ABU 524	<i>Bacillus simplex</i>	100 ^a
ABU 871	<i>Bacillus simplex</i>	93.3 ^b
ABU 882	<i>Bacillus megaterium</i>	100 ^a
ABU 890	<i>Bacillus simplex</i>	100 ^a
ABU 891	<i>Bacillus simplex</i>	91.2 ^b
ABU 1025	<i>Bacillus simplex</i>	100 ^a
Control	--	100 ^a

Means followed by a common letter within a column are not significantly different at $p \leq 0.05$

¹Sheath blight lesion spread was recorded at 5 days after incubation by Highest Relative Lesion Height method.

Table 6. Efficacy of PGPR strains on suppression of rice sheath blight in a detached leaf assay (Assay 5).

Strain	Identification	Sheath blight lesion spread ¹
ABU 1053	<i>Bacillus simplex</i>	87.5 ^{abc}
ABU 1240	<i>Bacillus mycoides</i>	66.6 ^c
ABU 1419A	<i>Bacillus simplex</i>	100 ^a
ABU 1627	<i>Bacillus mycoides</i>	75 ^{bc}
ABU 1645	<i>Bacillus simplex</i>	87.5 ^{abc}
ABU 1687	<i>Bacillus simplex</i>	88.7 ^{abc}
ABU 1930	<i>Bacillus simplex</i>	87.5 ^{abc}
ABU 1966	<i>Paenibacillus taichungensis</i>	90.8 ^{ab}
ABU 1970	<i>Bacillus simplex</i>	95.8 ^{ab}
ABU 2002	<i>Bacillus simplex</i>	95.8 ^{ab}
Control	--	100 ^a

Means followed by a common letter within a column are not significantly different at $p \leq 0.05$

¹Sheath blight lesion spread was recorded at 5 days after incubation by Highest Relative Lesion Height method.

Table 7. Efficacy of PGPR strains on suppression of rice sheath blight in a detached leaf assay (Assay 6).

Strain	Identification	Sheath blight lesion spread¹
ABU 2017	<i>Bacillus simplex</i>	85.4 ^b
ABU 2041A	<i>Bacillus simplex</i>	87.5 ^{ab}
ABU 2099B	<i>Bacillus simplex</i>	90.4 ^{ab}
ABU 2197	<i>Bacillus simplex</i>	97.0 ^a
ABU 2213	<i>Bacillus simplex</i>	91.6 ^{ab}
ABU 2252	<i>Bacillus megaterium</i>	95 ^{ab}
ABU 2424	<i>Bacillus simplex</i>	95.8 ^{ab}
ABU 2429B	<i>Bacillus megaterium</i>	87.9 ^{ab}
ABU 2549	<i>Bacillus mycoides</i>	66.6 ^c
ABU 2644	<i>Bacillus simplex</i>	90.8 ^{ab}
Control	--	100 ^a

Means followed by a common letter within a column are not significantly different at $p \leq 0.05$

¹Sheath blight lesion spread was recorded at 5 days after incubation by Highest Relative Lesion Height method.

Table 8. Efficacy of PGPR strains on suppression of rice sheath blight in a detached leaf assay (Assay 7).

Strain	Identification	Sheath blight lesion spread ¹
ABU 2772	<i>Bacillus subtilis</i>	45.8 ^{cd}
ABU 3099	<i>Bacillus simplex</i>	100 ^a
ABU 3118	<i>Bacillus simplex</i>	95.4 ^a
ABU 3128	<i>Bacillus simplex</i>	50 ^{cd}
ABU 3135	<i>Bacillus weihenstephanensis</i>	58.3 ^{bc}
ABU 3296	<i>Bacillus simplex</i>	65.8 ^b
ABU 3421A	<i>Bacillus vallismortis</i>	89.5 ^a
ABU 3454	<i>Bacillus weihenstephanensis</i>	41.6 ^d
ABU 3586	<i>Bacillus mycoides</i>	68.7 ^b
ABU 3819	<i>Bacillus aerophilus</i>	100 ^a
Control	--	100 ^a

Means followed by a common letter within a column are not significantly different at $p \leq 0.05$. Sheath blight lesion spread was recorded at 5 days after incubation by Highest Relative Lesion Height method.



Fig. 1. Sheath blight lesions induced by immature sclerotia of *R. solani* on rice in a detached leaf assay.



Fig. 2. Production of sclerotia from lesions caused by immature sclerotia in a detached leaf assay.



Challenged with MBI 600

Control

Fig. 3. Reduction of sheath blight lesions by *B. subtilis* MBI 600 in a detached leaf assay.

CHAPTER 4

PLANT GROWTH-PROMOTING ACTIVITIES OF *BACILLUS SUBTILIS*, MBI 600 AND ITS COMPATIBILITY WITH FUNGICIDES

ABSTRACT

Sheath blight (ShB) of rice caused by *Rhizoctonia solani* is an economically important disease, causing significant yield losses. The use of plant growth-promoting rhizobacteria (PGPR) to manage ShB is gaining popularity. In this study, the growth promoting activities of *Bacillus subtilis* MBI 600 such as production of IAA, cellulase, HCN, siderophores, and phosphate solubilization were assayed. The compatibility of strain MBI 600 with fungicides such as hexaconazole, propiconazole, validamycin, tricyclazole, benomyl, mancozeb, carbendazim, and azoxystrobin were evaluated. Strain MBI 600 was tested on four cultivars of rice such as Cocodrie, Catahoula, Neptune, and Trenasse under *in vitro* conditions for its influence on seedling growth. Seed bacterized rice seeds were placed in sterile beakers, covered with aluminum foil and incubated for 7 days, and the shoot and root lengths were measured. Rice cv. Cocodrie seeds were bacterized with strain MBI 600 at various concentrations (2.20×10^6 to 2.20×10^9 cfu/ml) and seeded in pots containing field soil and arranged on a GH bench in a randomized complete block design. Germination was recorded up to 7 days after sowing (DAS). Shoot and root lengths and weights were measured at 15 DAS and compared to non-bacterized control. The strain MBI 600 was found to produce siderophores. Seed bacterization with strain MBI 600 significantly increased shoot and root lengths at all concentrations in cvs. Cocodrie,

Catahoula, and Trenasse under *in vitro* conditions. The shoot lengths ranged from 39 to 42 mm at MBI 600 concentration of 2.20×10^9 cfu/ml in all rice CV's. Root lengths were significantly increased, compared to the control, at concentrations of 2.20×10^7 , 2.20×10^8 , and 2.20×10^9 cfu/ml. At a concentration of 2.20×10^9 cfu/ml, the root lengths in rice cvs. ranged from 47 to 69 mm. The shoot and root lengths of control seedlings were each up to 20 mm. Seed bacterization with 2.20×10^8 and 2.20×10^9 cfu/ml significantly increased seedling emergence (81 to 89%) compared to 2.20×10^6 and 2.20×10^7 cfu/ml, and non-bacterized control (61%) at 7 DAS under GH conditions. Similarly, seed bacterization with 2.20×10^9 cfu/ml of MBI 600 resulted in the highest shoot and root lengths (335 and 166 mm respectively), while the corresponding values for the non-bacterized control were 222 and 73 mm, respectively. Strain MBI 600 has shown good tolerance to hexaconazole, propiconazole, and validamycin; moderate tolerance to tricyclazole; and poor tolerance to benomyl and mancozeb at 1000 ppm. Strain MBI 600 showed compatibility to carbendazim and azoxystrobin up to 400 ppm. Overall, our results suggest that Integral produces siderophores, promoted rice seedling emergence and growth, and is compatible with rice fungicides.

INTRODUCTION

Rice is the major staple food crop for the majority of humans. However, production levels are reduced due to various fungal diseases. Among these diseases, sheath blight (ShB) caused by *Rhizoctonia solani* Kuhn. is a major production constraint causing significant yield losses under high input and high production environments worldwide (40). In U. S. rice growing regions of the Midsouth, ShB is the most devastating disease on rice (15, 23, 25). Conventional use of chemical fungicides for ShB management has negative effects on soil fertility, the ecosystem,

and increases crop production costs (9). Biocontrol of ShB using plant growth-promoting rhizobacteria (PGPR) offers a promising means of ShB management. PGPR strains are known to colonize and survive both in the rhizosphere and on the phyllosphere (21). In previous studies, use of PGPR has significantly improved growth and yields of rice (27). Their application promotes plant growth by direct and indirect mechanisms. Direct growth promotion is due to production of phytohormones, solubilization of phosphates (2, 20), increased uptake of iron through production of siderophores (9, 16), and volatile metabolites. Indirect methods of plant growth promotion are due to antibiosis, HCN (12), competition for space and nutrients, parasitism or lysis of pathogen hyphae, inhibition of pathogen-produced enzymes or toxins, and through induced systemic resistance (ISR) (31).

For a PGPR to be effective under field conditions, the key is to characterize the strain for plant growth-promoting and disease suppressing features. Moreover, knowledge on the exact mode of action is essential for devising effective disease management strategies (36). Research on rice ShB management through use of fresh cells (26, 47) or formulations of bacterial antagonists has been attempted (10, 49, 19). Seed emergence, plant growth promotion and increase in crop yields are the other attributes of a superior PGPR strain besides disease suppression. Earlier reports confirmed the enhancement of seed germination, seedling length, and dry matter production of roots and shoots of rice seedlings by PGPR (3).

In Asian countries, due to increased use of semi-dwarf, early-maturing, and high-yielding varieties, occurrence of ShB is common. The seriousness of ShB often warrants the use of chemical fungicides (45). Currently, ShB management is mostly through the use of systemic and non-systemic fungicides (33). Fungicides commonly used against ShB include Dithane M-45 (11), carbendazim (46), mancozeb (38), iprodione (18), and triazoles (44). Other fungicides such

as carbendazim and mancozeb as a mixture were also very effective (34). Among a new group of fungicides, strobilurins was highly effective both in terms of ShB control and rice grain yield enhancement (5). Application of fungicidal mixtures with more than one technical ingredient against multiple diseases is a common practice in rice production (43).

The compatibility of PGPR strains to fungicides is an important step for their use in ShB management. In earlier reports, use of *Pseudomonads* mixed with carbendazim and/or jinggangmycin, reduced ShB severity under greenhouse and field conditions (22, 52). In our earlier studies (Chapters II & III), *Bacillus subtilis* strain MBI 600 significantly suppressed mycelial growth, sclerotial germination of *R. solani*, and reduced ShB symptoms in rice under controlled conditions. The objectives of these studies were i) to characterize the strain MBI 600 for growth promoting traits, ii) to determine its effect on seedling emergence and growth of rice cultivars under *in vitro* and greenhouse conditions, and iii) to study its compatibility with fungicides in rice. The information gathered from these studies will be useful in devising management strategies against rice ShB.

MATERIALS AND METHODS

Source of rice cultivars. High yielding, conventional, long grain rice cultivars of Cocodrie, Neptune, Trenasse, and Catahoula developed at Rice Research Station, LSU AgCenter, Crowley, Louisiana, USA, were obtained and stored at 4⁰ C prior to use.

Source and production of *B. subtilis* MBI 600 in liquid formulation. The strain MBI 600 was obtained from the Phytobacteriology Laboratory strain collection, Department of Entomology and Plant Pathology, Auburn University, AL, USA. For laboratory and greenhouse studies, the liquid formulation of *B. subtilis* strain MBI 600 was produced by Becker Underwood

Inc., at their fermentation facilities located in Ames, Iowa, USA. The fermented product of MBI 600 was labeled as Integral®. The product contained a minimum of 2.2×10^{10} spores/ml. The product was packaged in 500 ml bottles and shipped to Department of Entomology and Plant Pathology, Auburn University, AL, USA to carry out the studies.

Purity check of *B. subtilis* strain MBI 600 in proprietary liquid formulation. To check for any cross contamination, the inoculum from bottles of Integral was streaked onto TSA plates and checked for growth and purity. This procedure was carried out in the laboratory of Prof. A. Podile in the Department of Plant Sciences, University of Hyderabad, Andhra Pradesh, India. To confirm the identity of MBI 600 strain, 16s rDNA sequence homology technique was used. Genomic DNA was isolated from the strain recovered from the product Integral by following standard procedures (1). Approximately, 1409 bp of the 16S rDNA was amplified by polymerase chain reaction (PCR) using the following primers: 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-ACG GCT ACC TTG TTA CGA CTT-3'). The resultant amplicon was verified by agarose gel electrophoresis. After verification of proper amplification, the amplicon was purified using a Qiagen Kit. The purified product was sequenced and the sequences were compared with known sequences in the databases using BLAST (basic logical alignment search tool).

A loopful of strain of MBI 600 stored in bottles was grown for 48 h at 25⁰ C in 20 ml sterile tryptic soy broth (TSB) (Difco, Detroit, Michigan, USA) on a reciprocating shaker (80 rpm). Bacterial suspension was centrifuged for 20 min at 10,000 x g. The resulting cell pellet was then washed two times in 0.1 M phosphate buffer (PB) (pH 6.8), resuspended in TSB amended with 20% sterile glycerol, and stored in vials at -80⁰ C prior to use. A new vial was used in each assay. The assays on characterization of MBI 600 strain for growth promotion were carried out

by utilizing the facilities at Department of Applied Botany and Biotechnology, University of Mysore, India.

Production of Indole Acetic Acid (IAA). Strain MBI 600 was retrieved from storage at - 80⁰ C, thawed and used for production of IAA. A loopful of inoculum was streaked onto TSA and incubated for 24 h. Single colonies were then inoculated into 250 ml flasks containing TSB and grown on a rotary shaker for 72 h. Liquid bacterial suspension were centrifuged at 3000 rpm for 30 min. Approximately, 2 ml of supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5M FeCl₃ solution). Production of IAA was confirmed through color indication as described by Brick *et al.* (7).

Phosphate solubilization. For phosphate solubilization assays, a medium containing 2 g yeast extract, 20 g glucose, 2 g tri-calcium phosphate, 60 mg actidione, and 15 g agar mixed with 1000 ml water, adjusted to pH 7, was used. A loopful of inoculum of strain MBI 600 was streak inoculated in the center of Petri dishes containing the media described above and incubated at 28⁰ C for 5 days and growth was observed. Bacterial colony forming clear zone was considered as phosphate solubilizer (37).

Production of siderophores. Chrome azurol S (CAS) assay was used to detect the production of siderophores by strain MBI 600. The composition of CAS agar was prepared by following the standard procedure (41). Pure culture of MBI 600 was stab inoculated on CAS agar plates using sterile toothpicks and incubated at 28⁰ C for 2 weeks in the dark. Development of an orange zone around bacterial growth was considered an indication of siderophore production. Reference bacterial strains with known siderophore production were used as positive controls. Plates of CAS-agar without strain of MBI 600 were incubated under the same conditions as

described above served as a control. Change of color in the CAS media is an indication of production of siderophore (9).

Production of HCN. Production of HCN by strain MBI 600 was determined by a modified method of Miller and Higgins (29). Pure culture of MBI 600 was streaked on to Petri dishes containing yeast extract mannitol agar (YEMA) amended with glycine (4.4 g/lit). Simultaneously, a filter paper soaked in 0.5% (w/v) picric acid in 1% Na₂CO₃ was placed in the upper lid of the Petri plate. After incubation at 28⁰ C for 4 days, color changes were examined. Development of an orange red color in YEMA is a characteristic of HCN production. Petri dishes containing YEMA without strain of MBI 600 served as control.

Production of Cellulase. Production of cellulase by strain MBI 600 was assessed in M9 medium (28) amended with yeast extract (1.2 g/L) and cellulose (10 g L⁻¹) and congo red (0.02%). Strain MBI 600 was spot inoculated in the center of Petri dish containing M9 media, and incubated for one week at 28⁰ C. Clear halos surrounding actively growing colonies are a positive sign for cellulose production (8).

Production of Chitinase. Chitinolytic ability of strain MBI 600 was assessed by streaking a loopful of 48-h-old culture of MBI 600 strain on water agar incorporated with 0.2% colloidal chitin (4). The plates were incubated at room temperature for 4 days. Development of a hydrolytic zone (clearing zone) around the actively growing colonies is a sign for chitinase production (50).

Effect of *B. subtilis* MBI 600 on seedling growth of various rice cultivars under *in vitro* conditions. Rice seeds of cvs. Cocodrie, Catahoula, Neptune and Trenasse, as described above were used for the current study. Rice seeds of each cultivar were surface sterilized in 2% sodium hypochlorite for 10 minutes and then were washed twice with sterile distilled water and air dried.

Two grams of surface sterilized rice seeds of each cultivar were soaked for 24 h in four different concentrations of strain MBI 600 produced in liquid formulation adjusted to 2.20×10^6 , 2.20×10^7 , 2.20×10^8 , and 2.20×10^9 cfu/ml. Seeds were air-dried in a laminar flow-hood. Seeds of each rice cultivar soaked in sterile distilled water served as non-bacterized control. Air dried seeds were incubated in sterilized 250-ml beakers covered with aluminum foil to prevent hydration and incubated at room temperature for 7 days. Root and shoot development were monitored daily. There were four replications for each cv and for each concentration of bacterial inoculum. Ten seedlings from each replicated treatment were sampled for shoot and root lengths. The root length was measured from the germination site to the end of the main root, and the shoot length was measured from the germination site to the highest tip of the shoot of each seedling.

Effect of *B. subtilis* strain MBI 600 on seedling emergence and growth under greenhouse conditions. Four concentrations of strain MBI 600 produced in liquid formulation were used to evaluate increases in emergence and growth of rice under greenhouse conditions. The concentrations used were 2.20×10^6 , 2.20×10^7 , 2.20×10^8 and 2.20×10^9 cfu/ml. One CV of rice, Cocodrie, was evaluated. Four grams of seed were soaked in different concentrations separately for 24 h and then air dried. Rice seeds soaked in sterile distilled water served as the control. Plastic pots filled with field soil were used to grow seedlings. There were six replications for each treatment, one pot per replication and 15 seeds were seeded at equi-distance at 2 cm depth in each pot. Seeded pots were arranged on a bench in the GH in a randomized complete block. Pots were maintained at 26 ± 2 °C and a RH of 90%. Rate of seedling emergence was recorded every day for 7 days. Root and shoot lengths and root and shoot weights were

recorded at 15 days after sowing (DAS). Individual seedlings were harvested and washed with tap water and air-dried. Shoot and root lengths and weights were measured.

Compatibility of *B. subtilis* strain MBI 600 to fungicides. Strain of MBI 600 produced in commercial liquid formulation was used for compatibility studies. The procedure described by Shanmugam and Narayanasamy (42) was implemented. Fungicides such as propiconazole (Tilt 250 EC), validamycin (Sheathmar 3L), benomyl (Benlate 50 WP), carbendazim (Bavistin 50 WP), tricyclazole (Beam 75 WP), mancozeb (80 WP), azoxystrobin (Heritage 50% WDG) and hexaconazole (Danzole 5 EC) were obtained from the manufacturers and were used for compatibility studies. Based on manufacturers' recommendations, the rates of 100, 200, 400, 600, 800, and 1000 ppm were selected. Nutrient agar (NA) plates amended with concentrations of fungicides were prepared by serial dilutions. Fresh culture of MBI 600 was retrieved from – 80⁰ C freezer and streaked on TSA plates. A loopful of active culture was streaked on individual NA plates amended with appropriate concentrations of fungicides and incubated for 48 h. There were five replications for each fungicide and concentration and one plate per replication. To measure the compatibility, growth of strain MBI 600 on fungicide amended media was rated as +++ (Good); ++ (Moderate); + (Poor); and – (No growth) and compared with growth of strain MBI 600 on non-amended fungicide NA plates.

Compatibility of strain MBI 600 to azoxystrobin and carbendazim was assessed according to the procedure described by Omar *et al.* (32). Fresh culture of strain MBI 600 was retrieved from -80⁰ C freezer and streaked on TSA plates. Purified single colonies were streaked on NA slants and incubated for 24 h at 30⁰ C. To this, 10 mL of sterile distilled water was added, and the bacterial culture was scraped from the agar surface with a sterile plastic loop. The bacterial suspension was homogenized by agitation using a vortex mixer. Sterilized YPG (yeast extract 5

g, bacterial peptone 5 g, glucose 20 g, 1000 ml H₂O, pH 6.8) liquid media were prepared and placed in 250 mL flasks. To these flasks, stock solutions of fungicides prepared in sterile distilled water at concentrations of 0, 200, and 400 ppm were added separately to make a final volume of 50 mL. The fungicide amended YPG media in flasks were later inoculated with 100µL of bacterial inoculum prepared as described above and incubated at 30⁰C at 250 rpm. The flasks were sampled every 24 h for 72 h and number of colony forming units was determined on NA using serial dilution. There were five replications for each concentration of fungicide. Media without fungicides served as controls.

Statistical analysis. The data were analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) and the treatment means were differentiated by a least significant difference (LSD) at P=0.05 using PROC- GLM.

RESULTS

Purity check of *B. subtilis* strain MBI 600 in proprietary liquid formulation. BLAST analysis of the 16s rDNA sequence of the strain MBI 600 generated from 1409 base pairs confirmed the purity and identity to the original identification of the parental strain prior to formulation in liquid.

Production of IAA, siderophores, cellulase, chitinase, HCN and phosphate solubilization by strain MBI 600. Strain MBI 600 was positive for siderophore production and negative for IAA, cellulose, chitinase, HCN and P solubilization (Table 1).

Effect of *B. subtilis* MBI 600 on seedling growth of various rice cultivars under *in vitro* conditions. Seed treatment with strain MBI 600 significantly increased shoot lengths compared to controls in cvs Cocodrie, Catahoula, and Trenasse (Table 2, Fig 2). At a concentration of 2.20

$\times 10^9$ cfu/ml, shoot lengths were highest in cvs Cocodrie, Catahoula, and Neptune. Shoot lengths were not significantly different at 2.20×10^9 and 2.20×10^8 cfu/ml for the cvs Trenasse. Similarly, shoot lengths were not significantly different for cv. Neptune at 2.20×10^8 and 2.20×10^7 cfu/ml of strain MBI 600. The shoot lengths in all rice cvs ranged from 39.1 to 41.5 mm at 2.20×10^9 cfu/ml, whereas in the control, the shoot lengths ranged from 7.6 to 19.5 mm.

Seed treatment with MBI 600 at 2.20×10^9 , 2.20×10^8 , and 2.20×10^7 cfu/ml significantly increased root lengths in all rice cvs over control (Table 3). At 2.20×10^9 cfu/ml, the root lengths in rice cvs ranged from 47.5 to 69.5 mm compared to control seedlings (8.3 to 19.9 mm). With increasing in concentrations of MBI 600, the root lengths were also increased in all four rice cvs. Development of mesocotyl roots and rootlets was prominent in all rice cvs at 7 days after incubation at 2.20×10^8 and 2.20×10^9 cfu/ml (Fig 2).

Effect of *B. subtilis* strain MBI 600 on seedling emergence and growth under greenhouse conditions. Seed treatment with all concentrations of strain MBI 600 significantly increased emergence of seedlings over control in rice cv Cocodrie from 5 days after seeding under greenhouse conditions (Table 4). However, in seed treatments with 2.20×10^8 and 2.20×10^9 cfu/ml of strain MBI 600, the emergence was significantly greater over controls from day 2 after seeding. The highest rate of germination (81 to 89%) was recorded at concentrations of 2.20×10^8 and 2.20×10^9 cfu/ml of strain MBI 600 at 7 days after seeding (Fig 3). The percent germination in non-bacterized control was 61%.

Shoot and root lengths were significantly longer in seed treatment with strain MBI 600 at 2.20×10^7 , 2.20×10^8 , and 2.20×10^9 cfu/ml over control (Table 5). At 2.20×10^9 cfu/ml the shoot and root lengths (Fig 4 and 5) were greatest (335 and 166 mm respectively) over controls (222 and 73 mm respectively). Shoot and root weights were significantly greater at a

concentration of 2.20×10^9 cfu/ml (0.23 and 0.10g). The shoot and root weights in non - bacterized control were 0.1 and 0.04 g, respectively.

Compatibility of *B. subtilis* strain MBI 600 to fungicides. Strain MBI 600 was compatible to 1000 ppm of hexaconazole, propiconazole, and validamycin based on its growth rated as good (Table 6). The strain was moderately compatible to tricyclazole and poorly compatible to benomyl and mancozeb at 1000 ppm. The strain has shown good compatibility up to 400 ppm when grown on YPG media amended with carbendazim and azoxystrobin. The strain has good compatibility to carbendazim (Fig 6) and azoxystrobin (Fig 7) at 400 ppm. The growth of strain MBI 600 in YPG media amended with carbendazim and azoxystrobin individually at 200 and 400 ppm was same as that of controls at 72 h after incubation (Fig 6 and Fig 7).

DISCUSSION

Various PGPR strains have been used to manage ShB disease and to enhance seedling growth and grain yields of rice (35, 48). To date, there have been no studies on mode of action of any particular PGPR strain used against ShB or used to improve rice seedling growth or yields of rice. In our present study, the strain MBI was found to be positive for siderophore production. Siderophores are low molecular weight iron chelating compounds produced by PGPR in soil and are known to suppress rice pathogens through siderophore mediated antibiosis (9). Under iron deprived conditions, *B. subtilis* secretes a catecholic siderophore termed as 2, 3-hydroxybenzoyl glycine that is similar to the precursor of *Escherichia coli* siderophore, enterobactin (14). Siderophore producing rhizobacteria have exhibited strong antagonism towards several rice pathogenic fungi such as *Alternaria* sp., *Fusarium oxysporum*, *Pyricularia oryzae* and *Sclerotium* sp. (9). Since iron is a limiting factor and is essential for the growth of microbes (17),

rhizobacteria develop strategies to acquire iron. Earlier studies showed that siderophore production is a key factor for a PGPR strain to control plant pathogens such as *R. solani* (30).

In these studies, the strain MBI 600 enhanced seedling emergence and growth of seedlings under laboratory and greenhouse conditions when used as seed treatment on various cultivars of rice. Significant enhancement of root and shoot growths was attributed to production of certain growth promoting substances and solubilization of elements such as phosphorus (Table 1). However, in our studies, the strain MBI 600 neither produced IAA nor solubilized phosphorus. Earlier reports showed that some strains of *B. subtilis* and *B. amyloliquefaciens* produced certain volatile compounds such as 2-3, butanediol and acetoin that stimulated plant growth (39). Production of gibberellins and cytokinins was also responsible for the physiological basis of growth promotion in rice seedlings. Growth promotion can also be due to indirect mechanisms such as ethylene inhibition through ACC deaminase activity (13). Further investigations are therefore needed in this direction to characterize the MBI 600 strain to identify the production of specific growth promoting substances involved in stimulating seed germination and promotion of rice seedling growth.

In our studies, the strain MBI 600 was highly tolerant to hexaconazole, propiconazole and validamycin; moderately tolerant to tricyclazole; and poorly tolerant to benomyl and mancozeb at 1000 ppm. The MBI 600 strain exhibited good tolerance at 400 ppm for carbendazim and azoxystrobin. Strains of *Bacillus* sp (B-44) were compatible to carbendazim at 500 and 1000 ppm respectively (22). The strain 916 of *B. subtilis* was found to colonize the root system successfully without any population decline when combined with Jingga mycin prior to application onto seed (52). Compatibility of strains of *Bacillus* spp. to strobilurins group of fungicides was also reported. Also, combined applications of *B. subtilis* strain NJ-18 with 50%

Kresoxim-methyl, strobilurin fungicide was very effective in suppressing rice ShB severity under field conditions (51). Use of fungicide-compatible PGPR strains in conjunction with fungicides offers better control than non-compatible strains. For example, the integration of Kodiak® (*Bacillus subtilis*) with fungicides as seed treatment significantly controlled seed and soil borne diseases of cotton under field conditions (6). Generally, seed bacterization with the higher inoculum concentrations yielded better growth promoting results than the lower inoculum concentrations on all the CVs of rice tested. In addition, strain MBI 600 showed compatibility to the majority of commonly used fungicides, which is a desired characteristic of PGPR strain. Hence, studies reported here suggest integration of strain MBI 600 with any of the fungicides that will have a commercial potential for management of ShB of rice under field conditions.

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Table 1. Plant growth promoting characterization of *Bacillus subtilis* strain MBI 600.

Character	Result ¹
Chitinase	-
IAA	-
Cellulase	-
Siderophore	+
HCN	-
P solubilization	-

¹ + = Positive; and - = Negative

Table 2. Effect of various concentrations of *Bacillus subtilis* strain MBI 600 as seed treatment on seedling growth of various rice cultivars under *in vitro* conditions.

Treatment ¹	Seedling height (mm) ²			
	Cocodrie	Catahoula	Neptune	Trenasse
Non bacterized control	7.6 ^c	17.6 ^e	10.9 ^c	19.5 ^d
2.20 x 10 ⁶ cfu/ ml	20.6 ^d	25.5 ^d	11.7 ^c	28.1 ^c
2.20 x 10 ⁷ cfu/ml	25.3 ^c	35.1 ^c	19.6 ^b	32.6 ^b
2.20 x 10 ⁸ cfu/ml	29.8 ^b	47.4 ^b	22.9 ^b	38.9 ^a
2.20 x 10 ⁹ cfu/ml	39.1 ^a	63.6 ^a	43.3 ^a	41.5 ^a

¹Seeds of rice treated with strain MBI 600 produced in liquid formulations at 2.20 x 10⁶, 2.20 x 10⁷, 2.20 x 10⁸, and 2.20 x 10⁹ cfu/ml.

²Means of four replications, 10 seedlings per replication

Means followed by a common letter in the columns are not significantly different according to LSD (at p≤0.05)

Table. 3. Effect of various concentrations of *Bacillus subtilis* strain MBI600 as seed treatment on root development of various rice cultivars under *in vitro* conditions

Treatment ¹	Root length (mm) ²			
	Cocodrie	Catahoula	Neptune	Trenasse
Non bacterized control	14.3 ^e	19.9 ^e	8.3 ^d	17.7 ^e
2.20 x 10 ⁶ cfu/ml	31.0 ^d	33.1 ^d	10.2 ^d	23.8 ^d
2.20 x 10 ⁷ cfu/ml	36.1 ^c	45.4 ^c	36.1 ^c	35.0 ^c
2.20 x 10 ⁸ cfu/ml	41.2 ^b	52.5 ^b	49.8 ^b	42.0 ^b
2.20 x 10 ⁹ cfu/ml	47.5 ^a	69.5 ^a	54.1 ^a	50.5 ^a

¹Seeds of rice treated with strain MBI 600 produced in liquid formulations at 2.20 x10⁶, 2.20 x 10⁷, 2.20 x 10⁸, and 2.20 x 10⁹ cfu/ml.

²Means of four replications, 10 seedlings per replication

Means followed by a common letter in the columns are not significantly different according to LSD (at p≤0.05)

Table 4. Effect of various concentrations of *Bacillus subtilis* strain MBI 600 as seed treatment on seedling emergence of rice (Cv. Cocodrie) under greenhouse conditions.

Treatment ¹	<u>Emergence of seeds (%)</u> ²				
	Day 2	Day 3	Day 4	Day 5	Day 6
Non-bacterized control	44.5 ^c	45.6 ^c	51.1 ^c	52.2 ^c	61.1 ^c
2.20 x 10 ⁶ cfu/ml	50.0 ^{bc}	50.0 ^{bc}	61.1 ^{bc}	65.6 ^b	73.3 ^b
2.20 x 10 ⁷ cfu/ml	50.0 ^{bc}	50.0 ^{bc}	68.9 ^{ab}	75.6 ^{ab}	78.9 ^b
2.20 x 10 ⁸ cfu/ml	54.4 ^{ab}	55.6 ^b	71.1 ^{ab}	75.6 ^{ab}	81.1 ^{ab}
2.20 x 10 ⁹ cfu/ ml	60.0 ^a	64.5 ^a	80.0 ^a	85.5 ^a	88.9 ^a

¹Seeds of rice treated with strain MBI 600 produced in liquid formulations at 2.20 x10⁶, 2.20 x 10⁷, 2.20 x 10⁸, and 2.20 x 10⁹ cfu/ml.

²Means of six replications, 15 seedlings per replication

Means followed by a common letter in the columns are not significantly different according to LSD (at p≤0.05)

Table. 5. Effect of various concentrations of *Bacillus subtilis* MBI 600 as seed treatment on growth of rice seedlings (Cv. Cocodrie) under greenhouse conditions at 15 days after seeding.

Treatment	Shoot height (mm)¹	Root length (mm)¹	Shoot fresh weight (g)¹	Root fresh weight (g)¹
Non bacterized control	222.0 ^c	72.7 ^d	0.10 ^c	0.04 ^b
2.20 x 10 ⁶ cfu/ml	234.3 ^c	95.7 ^c	0.10 ^c	0.06 ^b
2.20 x 10 ⁷ cfu/ml	289.0 ^b	119.0 ^b	0.14 ^{bc}	0.07 ^{ab}
2.20 x 10 ⁸ cfu/ml	298.7 ^b	132.3 ^b	0.16 ^b	0.07 ^{ab}
2.20 x 10 ⁹ cfu/ml	335.0 ^a	166.3 ^a	0.23 ^a	0.10 ^a

¹Seeds of rice treated with strain MBI 600 produced in liquid formulations at 2.20 x 10⁶, 2.20 x 10⁷, 2.20 x 10⁸, and 2.20 x 10⁹ cfu/ml.

²Means of six replications, 15 seedlings per replication

Means followed by a common letter in the columns are not significantly different according to LSD (at p≤0.05)

Table 6. Compatibility of *B. subtilis* strain MBI 600 with commonly used fungicides.

Fungicides	Fungicide concentrations (ppm) ¹					
	100	200	400	600	800	1000
Propiconazole	+++	+++	+++	+++	+++	+++
Validamycin	+++	+++	+++	+++	+++	+++
Benomyl	+++	+++	+++	+++	++	+
Carbendazim	+++	+++	+++	+	-	-
Tricyclazole	+++	+++	+++	+++	+++	++
Mancozeb	+++	+++	+++	++	+	+
Azoxystrobin	+++	+++	+++	+	-	-
Hexaconazole	+++	+++	+++	+++	+++	+++

¹Rate of growth of strain MBI 600 in nutrient agar amended with various concentrations of fungicides: +++ = Good; ++ = Moderate; + = Poor; and - = No growth.



Bacterized seed with MBI 600

Non bacterized control

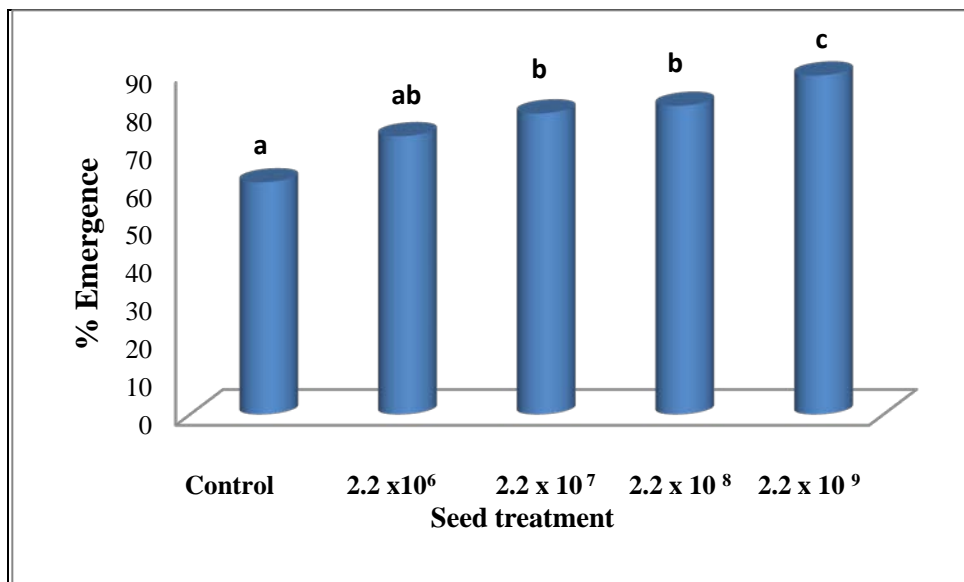
Fig. 1. Influence of strain MBI 600 as seed treatment at 2.2×10^9 cfu/ml on growth of rice seedlings (CV. Cocodrie) under laboratory conditions at 7 days after seeding.



Bacterized seed with MBI 600

Non bacterized control

Fig. 2. Influence of strain MBI 600 as seed treatment at 2.2×10^9 cfu/ml on growth of mesocotyl roots and rootlets of rice seedlings of CV. Cocodrie at 7 days after seeding.



Values are means of six replications, 15 seeds per replication

Means followed by a common letter are not significantly different according to LSD (at $p \leq 0.05$)

Fig. 3. Influence of various concentrations of *Bacillus subtilis* strain MBI 600 as seed treatment on seed germination of rice, CV. Cocodrie, at 7 days after seeding under greenhouse conditions.



A=Non bacterized control, B= 2.20×10^6 cfu/ml, C= 2.20×10^7 cfu/ml, D= 2.20×10^8 cfu/ml and E= 2.20×10^9 cfu/ml

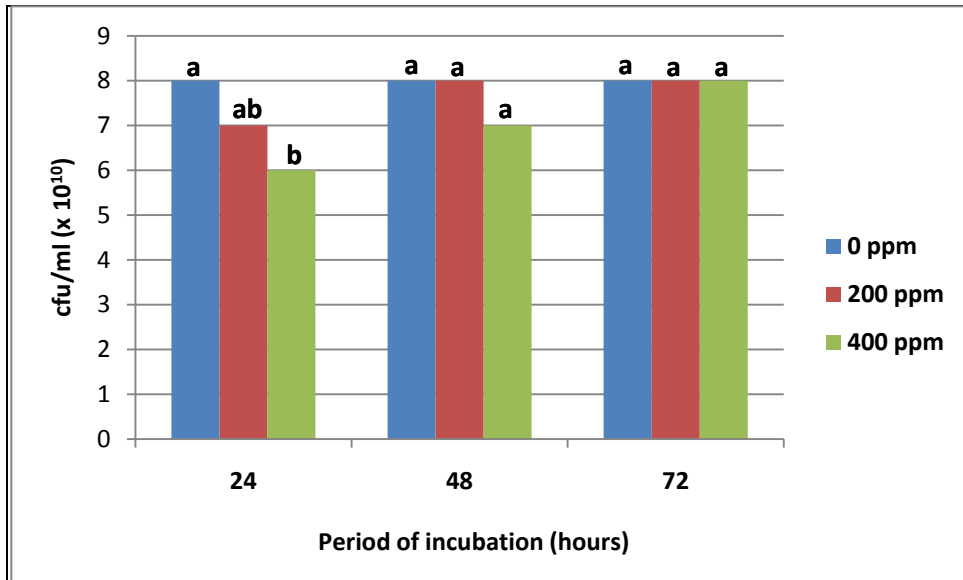
Fig 4. Influence of various concentrations of *Bacillus subtilis* strain MBI 600 as seed treatment on root growth of rice under greenhouse conditions.



Non bacterized control

Seed bacterized with MBI 600
at 2.20×10^9 cfu/ml

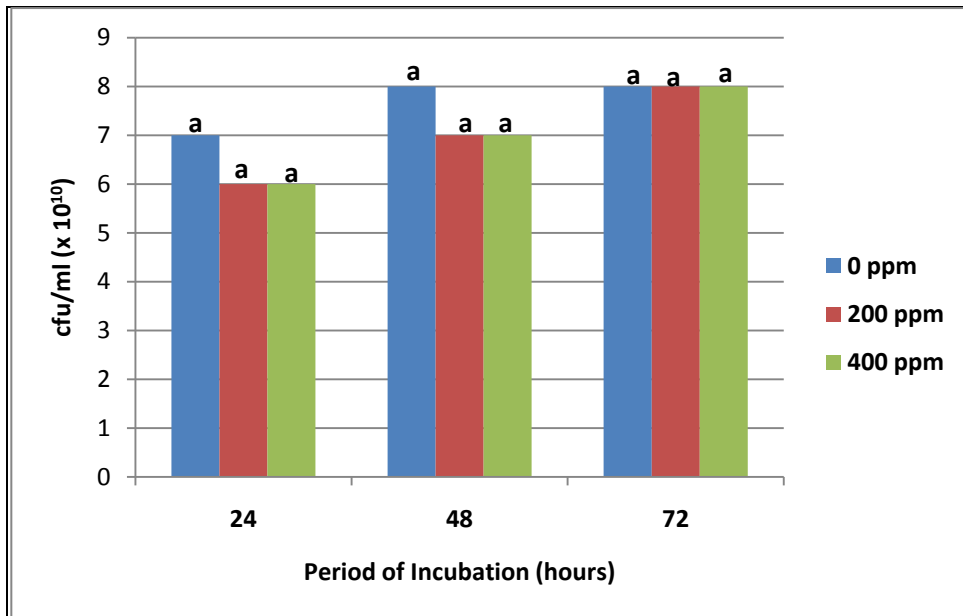
Fig. 5. Influence of various concentrations of *Bacillus subtilis* strain MBI 600 as seed treatment on seedling growth of rice under greenhouse conditions.



Values are means of five replications, one plate per replication

Means followed by a common letter are not significantly different according to LSD (at $p \leq 0.05$)

Fig. 6. Growth of strain MBI 600 on nutrient agar amended with various concentrations of carbendazim.



Values are means of five replications

Means followed by a common letter are not significantly different according to LSD (at $p \leq 0.05$)

Fig. 7. Growth of strain MBI 600 on nutrient agar amended with various concentrations of Azoxystrobin.

CHAPTER 5

EFFICACY OF *BACILLUS SUBTILIS* MBI 600 (INTEGRAL®) AGAINST SHEATH BLIGHT CAUSED BY *RHIZOCTONIA SOLANI*, ON GROWTH AND YIELD OF RICE

ABSTRACT

Sheath blight (ShB) disease assay was developed to screen Integral against ShB. To develop an assay, rice CV. Swarna seedlings were raised in 30 CM pots containing field soil with Osmocote fertilizer. Leaf sheaths of 30-days-old seedlings were inoculated near base with either one of the immature, mature and aged sclerotia of *R. solani* produced on PDA. Inoculated seedlings were incubated under greenhouse conditions, and developing lesions of ShB were rated at 4-days after inoculation. The efficacy of Integral on rice seedling growth and its potential for suppression of ShB were evaluated under greenhouse conditions. In this, four concentrations of Integral (2.20×10^6 , 2.20×10^7 , 2.20×10^8 and 2.20×10^9 cfu/ml) were used as seed treatment (ST) and seedlings were raised in pots. Seedling germination and seedling height were taken. Seedlings raised from seed treatment with above four concentrations were treated as a root dip (SD) again with respective concentrations to provide additional boost of Integral application. Dip-treated seedlings were transplanted into 30 CM pots containing field soil. At 30 days after transplanting (DAT), leaf sheaths of each seedling were inoculated with immature sclerotia for ShB disease development. Again at 45 DAT, every seedling in respective treatments was treated with respective concentrations of Integral as a foliar spray (FS) until run-off to provide another additional boost. Seedlings treated with carbendazim at 1g/L served as chemical control.

Seedlings treated with water served as non-bacterized control. Overall, there were 10 treatments; each treatment was replicated five times, one plant per pot. Pots were arranged in a Randomized Complete Block Design and maintained in greenhouse at 26⁰ C, RH of 90%, and a photoperiod of 16 h. ShB disease severity was rated at 52 DAT and seedling height and tillers were taken at 60 DAT. Efficacy of Integral at two concentrations (2.20×10^8 and 2.20×10^9 cfu/ml) was evaluated in two identical field trials during 2009 on rice CV. Swarna against ShB, growth of seedlings, and grain yield. Initially, seedlings were produced in a nursery bed with bacterized seed treatment. Seedlings of 30-days-old were treated with Integral at above concentrations as a root dip and transplanted into 10 m² blocks of flooded field. Again at 45 and 60 DAT, Integral was applied as a FS as described above. Carbendazim was used as a chemical control. There were 10 treatments, and each treatment was replicated eight times, and arranged as a factorial RCBD. At 20 DAT, each treatment plot was broadcast inoculated with *R. solani* inoculum produced on rice grains for uniform ShB development. Seedling height before transplanting, ShB severity at 90 DAT, and grain yield were taken at harvest. In ShB disease assay, immature sclerotia provided excellent ShB lesions compared to mature and aged sclerotia. Integral at 2.20×10^9 cfu/ml provided significant increase of seedling shoot height and root length compared to other concentrations and control under GH conditions. Similarly, at 2.20×10^9 cfu/ml, Integral significantly suppressed ShB severity compared to other concentrations and non-bacterized control, and comparable to chemical control. Similarly, Integral provided significant increase of seedling height in nursery, production of tillers at a similar level in both field trials as ST+SD+FS, compared to its lower concentrations and non-bacterized control. Also, ShB disease severity was significantly suppressed with higher concentrations of Integral compared to lower concentrations. Grain yields were significantly better with higher concentrations of Integral

compared to lower concentrations, non-bacterized control and chemical control. Overall, our results suggest that Integral significantly reduced ShB severity, enhanced seedling growth, tillers/plant and grain yields at a concentration of 2.2×10^9 cfu/ml as seed treatment, root dip and foliar sprays under the conditions evaluated.

INTRODUCTION

Sheath blight (ShB) of rice is an economically important disease in all crop growing areas of the world. Significant grain yield losses were reported due to ShB when susceptible varieties are grown (26). The disease is caused by a soil-borne fungal pathogen, *Rhizoctonia solani* Kuhn. The pathogen survives as sclerotia and mycelia in plant debris, and on weeds in the tropics (15). In temperate regions, the primary source of inoculum is sclerotia produced in previous rice crops (16). Strong sources of genetic resistance are not available for ShB, and the disease is currently managed through use of chemical fungicides (22). Fungicidal management of ShB often gives inconsistent results and is not economical. Indiscriminate use of fungicides and chemical fertilizers to increase rice yields has several concerns relating to environmental hazards, pathogen resistance, leaching losses, and destruction of beneficial microflora. Use of plant growth-promoting rhizobacteria (PGPR) as biocontrol agents is gaining popularity in managing rice diseases and in enhancing growth and grain yields (18).

Soil bacteria in rice ecosystems have been shown to have exhibit significant fungistasis on vegetative growth and sclerotia of *R. solani* (45). Application of PGPR to control ShB under field conditions was attempted earlier (17, 7, 11). *Bacillus* spp. have been used in biocontrol of ShB. *Bacillus* inoculants tolerate desiccation, heat, oxidizing agents and UV radiations compared to Gram negative bacteria (10). The *Bacillus* spp. causes reduction in pathogen inoculum at

infection site due to antibiosis, competition for space and nutrients, inhibition of pathogen related enzymes or toxins, parasitism or lysis of pathogen hyphae, and through induced systemic resistance (4, 39). In addition, plant growth promotion by *Bacillus* spp. is also elicited through increased N uptake, phosphate solubilization, siderophore and phytohormone production. Strains of *B. subtilis* and *B. megaterium* have shown significant inhibition of *R. solani* (45). Enhanced plant growth and grain yields in rice with *Bacillus* spp. application were also well documented (29, 28, 2, 39).

Several PGPR formulations have been evaluated for management of rice ShB. Most *Bacillus* formulations that were tested included bacterial cell suspensions (39), water soluble granules, floating pellets (12), powder formulations, and empty fruit bunch powders (EFB) (2). The field efficacies of these formulations were not consistent due to varied reasons. The survival rates and application efficiencies of PGPR generally are dependent on variations in the microclimate of a crop. Further, the field efficacy of a commercial product of PGPR is dependent on its shelf life, delivery at appropriate dose, type of formulation used, and available concentration of PGPR. The time of application of PGPR can also affect their efficacy in managing ShB (44). Since *R. solani* is a soil-borne pathogen that will eventually spread to leaf sheath and blades, effective management of the ShB necessitates bacterial application to seeds (17), roots (2), or foliage (12). Synergistic effects in ShB management can be attained by combined applications of PGPR to seeds, roots, and foliage (28).

Optimization of a ShB assay under greenhouse conditions is a pre-requisite for evaluating PGPR. Several inoculation procedures using different inoculum sources of *R. solani* have been tried (6, 34, 24). These include *R. solani* colonized tooth picks (30, 31, 47), agar plugs (8, 13), rice grain-hulls (14, 23, 42), mycelia fragments (32, 40) sclerotia (34), and liquid cultured

mycelia balls and suspensions (24). However, none of these assays that involved greenhouse conditions have addressed their use for screening of PGPR strains.

In our earlier studies, we have screened 70 PGPR strains with known efficacies on other crops and pathogens. As shown in our previous chapters, the majority of the strains showed significant responses against ShB. Specifically, Integral significantly suppressed mycelial growth, sclerotial germination, and reduced ShB symptoms caused by *R. solani* under laboratory assays (Chapter 2, and 3). Integral was found to produce siderophores and enhanced rice seed germination and seedling growth under both laboratory and greenhouse conditions. Furthermore, Integral was compatible with commonly used fungicides in rice (Chapter 4). Based on our previous work, the objectives of the present study were i) to develop a reliable ShB assay to screen various concentrations of Integral against ShB, ii) to screen various concentrations of Integral for suppression of ShB, improving seedling growth under GH conditions, and iii) to test the efficacy of Integral in field trials against ShB and grain yield of rice.

MATERIALS AND METHODS

Source of pathogen and production of sclerotia of *Rhizoctonia solani*. A multinucleate and virulent isolate of *R. solani* belonging to anastomosis group AG-1 IA was obtained from the culture collection of Dr. S. Krishnam Raju, Andhra Pradesh Rice Research Institute (APRRI), India. The isolate was originally isolated from ShB infected rice seedlings. The culture was maintained on potato dextrose agar (PDA) for further use. For production of sclerotia, *R. solani* was grown on PDA at $28\pm 1^{\circ}\text{C}$ in the dark. The sclerotia were harvested at different time intervals and categorized according to their age as follows: immature (<5-day-old), mature (5-30 day-old) and aged (>30-days-old). The selected sclerotia were stored at 4°C prior to use.

Source of rice cultivar. Rice seeds of CV Swarna, developed at Andhra Pradesh Rice Research Institute (APRRI), India, were obtained and used in the present study. Swarna is a potentially high-yielding, long duration crop (150 days) with bold and golden yellow colored grains, and is extremely susceptible to ShB. The seeds were stored at 4⁰ C prior to use.

Source and production of *B. subtilis* MBI 600 in liquid formulation. For greenhouse and field studies, the liquid formulation of *B. subtilis* strain MBI 600 was produced by Becker Underwood Inc., at their fermentation facilities located in Ames, Iowa, USA. The formulated product of MBI 600 in liquid was labeled as Integral®. The product contained a minimum of 2.20 x 10¹⁰ spores/ml and was packaged in 500 ml bottles and shipped to APRRI, India to carry out studies described here.

Optimization of a ShB assay under greenhouse conditions. Rice seeds of CV. Swarna were soaked in water for 24 h. Rice seedlings were produced in 30 CM diameter pots by sowing two seeds per pot containing field soil amended with Osmocote fertilizer. Pots were initially filled with tap water and the soil was soaked completely for 72 h. Later, the soil was agitated manually to break the aggregates and the excess water was drained. The pots were maintained at a temperature of 26 ± 2⁰ C and RH of 90% in a randomized complete block design on a bench in the GH. Pots were placed in plastic trays under submerged conditions after 4th leaf stage.

Immature, mature, and aged sclerotia of *R. solani* produced on PDA as described above were used as a source of pathogen inoculum. For inoculation, sheaths of second expanded leaves of 30-day-old plants were selected. The leaf sheaths at 2 cm above water line were opened carefully and an immature, mature and aged sclerotia were inoculated individually by placing one sclerotium per plant (34), The inoculated portion was sealed with cellophane tape and watered immediately. Plants not inoculated with sclerotia of any type served as controls. There were five

replications for each sclerotial type and one plant per replication. Inoculated pots were maintained under the same conditions as above for another seven days. Observations of ShB lesions caused by different aged sclerotia on the leaf sheaths were made, and the size of the lesions was measured at 4 days after inoculation. The incubation period was calculated as the time taken from inoculation to 50% appearance of water soaked lesions of ShB (46). The lesion length around the sclerotium was measured, and lesion size was assessed by multiplying the length and width of lesions. ShB severity was rated according to Relative Lesion Height (RLH) method (33) with the following formula:

$$\% \text{ RLH} = 100 \times \text{Total height of lesions} / \text{Total plant height}$$

Efficacy of Integral on Sheath blight and growth of rice seedlings under greenhouse conditions. The efficacy of Integral on ShB severity and seedling growth of rice was tested under GH conditions (12, 21, 38) by adopting the following procedure. Four concentrations of Integral (2.20×10^6 , 2.20×10^7 , 2.20×10^8 and 2.20×10^9 cfu/ml) were selected for testing. The concentrations of Integral were introduced onto rice seeds as seed treatment (ST); ST + seedling root dip (SD); and foliar sprays (FS). For seed treatment, seeds of rice were surface sterilized with 2% sodium hypochlorite for 5 min, and washed with sterile distilled water two times. Surface sterilized seeds were soaked in four concentrations of Integral as described above for 24 h, separately. Seeds were later removed from the bacterial soaked solutions and air dried in a laminar flow hood for 30 min. Seeds were planted into 30 CM diameter plastic pots containing field soil collected from paddy fields. The soil is typical deltaic alluvial with a pH of 7.2. There were 10 seeds per pot. Carbendazim (1g/L) treated seeds served as a standard chemical control. Seeds soaked in water served as non-bacterized controls. There were six treatments, five replications per treatment, with one pot per replication. Replicated pots were arranged on a

greenhouse bench in a RCBD fashion. The pots were maintained at $26\pm 2^{\circ}$ C, RH of 90% and photoperiod of 16 h and grown for 25 days. Germination was observed after 7 days post seeding. Seedling growth parameters such as root and shoot lengths were taken at 25 days. Later, 25-days-old seedlings treated with four concentrations of Integral were transplanted into 30 CM diameter pots, 2 seedlings per pot containing same field soil, after dipping with Integral at appropriate concentrations to boost inoculation. For dipping, roots of seedlings were soaked in Integral for 4 h. Seedlings soaked in water were used as non-bacterized controls.

Since immature sclerotia of *R. solani* gave optimum level of ShB lesions in optimization studies, they were used to inoculate the 30-day-old transplanted seedlings. Treated seedlings were artificially inoculated with immature sclerotia, near the base of leaf sheath above water level to get an optimum level of ShB disease to evaluate the efficacy of Integral against ShB. At 15-days post pathogen inoculation, Integral was applied again as a foliar spray onto transplanted seedlings with four concentrations, and treated as a separate set of treatments. For foliar sprays, 25 ml of Integral at appropriate concentrations were sprayed on seedlings at 45 DAT using a back pack sprayer until run-off. The following treatments were included:

- 1) ST + SD with Integral at 2.20×10^6 cfu/ml
- 2) ST + SD with Integral at 2.20×10^7 cfu/ml
- 3) ST + SD with Integral at 2.20×10^8 cfu/ml
- 4) ST + SD with Integral at 2.20×10^9 cfu/ml
- 5) ST + SD + FS with Integral at 2.20×10^6 cfu/ml
- 6) ST + SD + FS with Integral at 2.20×10^7 cfu/ml
- 7) ST + SD + FS with Integral at 2.20×10^8 cfu/ml
- 8) ST + SD + FS with Integral at 2.20×10^9 cfu/ml

9) ST + SD + FS with carbendazim at 1g/L

10) Non-bacterized control.

Each treatment was replicated five times and replicated pots were arranged on a greenhouse bench in a RCBD, and maintained at 26 ± 2^0 C, with a RH of 90%, and photoperiod of 16 h. Pots were fertilized with NPK (1.5-0.5-0.5g/pot) at the time of pathogen inoculation. Other agronomic practices were followed according to guidelines of APRRI to maintain the seedlings. Seedling height and number of tillers per plant were taken at 60 DAT. ShB disease severity was assessed at 52 DAT as described previously.

Efficacy of Integral on rice ShB, growth of seedlings, and yield under field conditions.

Field studies were conducted at Andhra Pradesh Rice Research Institute, Maruteru, A. P., India during rainy season (July to November) of 2009. APRRI is a leading center for rice research in India. It is located in the typical deltaic region of Andhra Pradesh at a Latitude of 16.380 N and Longitude of 81.440 E, at an altitude of 5 m above mean sea level. The soils are typical deltaic alluvials with pH of 7.2. The experimental site is known for its occurrence of ShB inoculum due to continuous rice cultivation and is designated as a ShB sick field. There were two field trials, 1 km away from each other. Two identical field trials were conducted to minimize the risk of losing a trial in case of flooding due to rains or non-occurrence of disease. The trials were arranged in factorial RCBD. Integral was evaluated at two concentrations (2.20×10^8 and 2.20×10^9 cfu/ml), since Integral has provided very good efficacy results under greenhouse conditions against ShB. Integral was used as ST at time of sowing in the nursery to produce seedlings for field transplanting.

Production of seedlings in nursery. For production of seedlings, nursery beds were prepared as follows. Only one nursery bed was used to produce seedlings for two field trials. To

prepare nursery bed, the soil was ploughed, puddled with water and leveled. The puddle mud was later allowed to settle down and the excess water was removed. The nursery area was divided into beds to accommodate various seed treatments. Each bed was 2.5 m wide x 4.0 m in length. NPK was applied at the rate of 0.5-0.5-0.5 kg/100 m² to nursery area. Prior to sowing into nursery beds, rice seeds were treated with Integral at two concentrations (2.20×10^8 and 2.20×10^9 cfu/ml) separately. Carbendazim was used as the standard chemical control. Seeds soaked in water served as non-bacterized controls. Treated seeds were sown on to nursery beds by broadcasting at the rate of 50 kg/ha. There were four treatments in the nursery and one bed per treatment. The treatments were as follows: i) ST with Integral at 2.20×10^8 cfu/ml; ii) ST with Integral at 2.20×10^9 cfu/ml; iii) ST with carbendazim at 1g/L; and iv) non-bacterized control. Another dose of 0.5 kg N was applied at 12 days after seeding in the nursery beds. Agronomic practices for rice nursery management developed by APRRI, India were followed. At 30-days after seeding, twenty seedlings from each treatment were pulled, washed with water and air dried, after which shoot and root lengths were measured.

Field site preparation and maintenance of transplanted crop. The experimental area intended for transplanting was flooded with water and ploughed until all soil aggregates were broken up. The excess water was drained after 48 h and the site was partitioned manually into 8 main blocks. Each main block was divided into seven sub-plots of 10 m² each to accommodate various treatments. Each individual sub-plot included earth embankments to prevent water movement among the treatments. Seedlings were pulled from appropriate treatments in nursery beds at 30-days after seeding and were separately grouped into bundles for ease of transplant. Prior to transplanting, seedling roots were dipped in Integral at concentrations of 2.20×10^8 and 2.20×10^9 cfu/ml, separately, for 6 h. Seedlings dipped in carbendazim at 1g/L served as

standard chemical control, whereas seedlings dipped in water served as non-bacterized dipped control. Seedlings were then transplanted into sub-plots at a spacing of 15 x 15 cm². The transplanted area remained in a submerged condition until harvest. To ensure uniform ShB incidence, *R. solani* multiplied on rice grains were broadcast applied into the field at 20 DAT. NPK was applied at a rate of 80-40-30 kg/ha as follows. Phosphorus and potassium fertilizers were applied as basal prior to transplanting, whereas nitrogen was applied at 3 stages equally at basal, active tillering, and panicle initiation stages. Again, two foliar sprays with Integral at 2.20 x 10⁸ and 2.20 x 10⁹ cfu/ml were applied at 45 and 60 DAT onto plants already treated with Integral as ST and SD treatments. Carbendazim (1g/L) was sprayed again on carbendazim treated plants and water was sprayed on water control plants. The following treatments were included.

1. ST + SD with water (non-bacterized control)
2. ST + SD + FS with water (non-bacterized control)
3. ST + SD with Integral at 2.20 x 10⁸ cfu/ml
4. ST + SD + FS with Integral at 2.20 x 10⁸ cfu/ml
5. ST + SD with Integral at 2.20 x 10⁹ cfu/ml
6. ST + SD + FS with Integral at 2.20 x 10⁹ cfu/ml
7. ST + SD + FS with Carbendazim at 1g/L

There were 8 replications for each treatment.

Measurement of seedling growth. Ten seedlings from each replication of transplanted plots in appropriate treatments were carefully pulled at 60 DAT and plant height and tillers per plant were taken. Plant heights were measured from the collar region to the main tip of each seedling. Number of tillers for each plant were counted from the unelongated basal internodes.

Disease assessment. At 90 DAT, seedlings were rated for ShB severity from appropriate treated replicated plots. There were 10 seedlings per replication. Percent diseased tillers were calculated by comparing the number of diseased tillers to the total tillers in a plant. The height of the ShB lesion from plant base was measured and disease severity was calculated by RLH method as described above by using the following formula:

$$\% \text{ RLH} = 100 \times \text{Total height of lesions} / \text{Total plant height}$$

Assessment of yield. Seedlings from each treatment were manually harvested for grain yield. Total seedlings from individual replicated plots were collected at 120 DAT, bundled, and dried on site for 2 days. The dried plants were later moved to a threshing floor, and threshed manually for grain separation. Collected grains were stored, dried, and weighed.

Statistical analysis. The data were analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) and the treatment means were differentiated by a least significant difference (LSD) at $P=0.05$ using PROC- GLM.

RESULTS

Optimization of a ShB assay under greenhouse conditions. Inoculation of leaf sheaths with different ages of sclerotia provided ShB lesions on leaf sheaths to various degrees. Lesion size was highest with immature sclerotia (1.1 cm^2) and was significantly superior over that of mature (0.8 cm^2) and aged sclerotia (0.7 cm^2) (Table 1) (Fig. 1). The ShB severity ranged from 28.7 to 30.6% with different aged sclerotia, and no significant differences were noticed at 4 days post inoculation. The incubation period was least for immature sclerotia (48 h) and is less compared to that of mature and aged sclerotia (60 h each).

Efficacy of Integral on Sheath blight and growth of rice seedlings under greenhouse conditions. Seed treatment with concentrations of Integral at 2.20×10^8 and 2.20×10^9 cfu/ml significantly increased seedling germination compared to control at 7 days after seeding (Fig. 2). The highest rate of germination (95.6%) was obtained with a concentration of 2.20×10^9 cfu/ml. Seed treatment with carbendazim gave 90.8% seedling germination. The germination in non-bacterized control was 88%. Root lengths on 25-day-old seedlings were significantly better in seed treatment with Integral at 2.20×10^9 cfu/ml (12.2 cm) and 2.20×10^8 cfu/ml (9 cm) over control. Shoot lengths were higher (40.7 cm) at 2.20×10^9 cfu/ml than others. The root and shoot lengths in non-bacterized control were 7.9 and 33.8 cm, respectively (Fig. 3).

Plant height and tillers per plant at 60 DAT were significantly enhanced in all treatments with Integral compared to control (Table 2). Plant heights were highest at 2.20×10^9 cfu/ml as ST + SD + FS (73.2 cm). Integral gave better plant height (70.8 cm) as ST + SD + FS at 2.20×10^8 cfu/ml. Plant height with carbendazim as ST + SD + FS was 58.9 cm and was not significant over non-bacterized control (58.3 cm). Number of tillers per plant was higher at a concentration of 2.20×10^9 cfu/ml as ST + SD + FS (11.9), and as ST + SD (11.6) with no significant differences between them. At 2.20×10^8 cfu/ml, the number of tillers was 9.6 per plant, whereas 6.3 in non-bacterized control. ShB lesions were significantly reduced with all concentrations of Integral (Table 2). ShB severity was least at a concentration of 2.20×10^9 cfu/ml as ST + SD + FS (9.2%), and with carbendazim (7.9%). ShB severity was up to 24.1% with 2.20×10^8 cfu/ml, whereas 65.6% in non-bacterized control (Fig. 4).

Efficacy of Integral on rice ShB, growth of seedlings, and yield under field conditions. Seed treatment with Integral significantly improved root and shoot lengths of 30-day-old seedlings compared to control in nursery (Fig. 5). Root lengths were highest at concentrations of

2.20×10^9 and 2.20×10^8 cfu/ml (14 and 9.3 cm respectively) with no significant differences among them. Shoot lengths were highest at 2.20×10^9 cfu/ml (44.9 cm) compared to 2.20×10^8 cfu/ml (37 cm). Carbendazim seed treatment significantly improved root length (9.6 cm) over control. The root and shoot lengths were about 8.4 and 36 cm respectively in non-bacterized control.

On a transplanted crop, application of various concentrations of Integral significantly reduced diseased tillers per plant, and ShB severity compared to control in both the field trials (Table 3) (Fig. 6). The mean diseased tillers per plant were least with carbendazim (29.0%), followed by Integral at a concentration of 2.20×10^9 cfu/ml as ST + SD + FS (31.9%). However, there were no significant differences between carbendazim and Integral at 2.20×10^9 cfu/ml as ST + SD + FS in Trial 1. The mean diseased tillers were 53.2% with a concentration of 2.20×10^8 cfu/ml. In non-bacterized control, the mean diseased tillers were at 97.1%. Mean ShB severity was lowest in carbendazim treated plots (18.3%), followed by plots applied with Integral at a concentration of 2.20×10^9 cfu/ml as ST + SD + FS (22.9%). However, the efficacy of Integral as ST + SD + FS at 2.20×10^9 cfu/ml was similar with carbendazim in Trial 1. The mean ShB severity at 2.20×10^8 cfu/ml was 27% and 65.3% in non-bacterized control.

Plant height and tillers per plant were significantly better in treatments with both concentrations and methods of application of Integral compared to control (Table 4). Mean plant heights were highest at a concentration of 2.20×10^9 cfu/ml of Integral as ST + SD + FS (96.9 cm). At 2.20×10^8 cfu/ml, plant heights were 93.1 cm and 82.7 cm in non-bacterized control. Similarly, tillers were highest with Integral at 2.20×10^9 cfu/ml (12.7 per plant) followed by Integral at 2.20×10^8 cfu/ml (11.6 per plant). The tillers in non-bacterized control were 10.3 per plant.

Grain yield was significantly enhanced with different concentrations and methods of Integral application (Table 5). Grain yield was highest with Integral at 2.20×10^9 cfu/ml as ST + SD + FS (6065 kg/ha). Next best grain yields were obtained with ST + SD of Integral at 2.20×10^9 cfu/ml (5650 kg/ha). Integral at 2.20×10^8 cfu/ml as ST + SD + FS also produced significant yields up to 5376 kg/ha. Mean grain yield in carbendazim treated plots were about 5507 kg/ha. In non-bacterized control, the grain yield was 4129 kg/ha. Grain yield increase over control was highest with application of Integral as ST + SD + FS (46.9%), followed by ST + SD (36.8%) at a concentration of 2.20×10^9 cfu/ml (Fig. 7). Integral at 2.20×10^8 cfu/ml produced a yield increase of 30.2%. Carbendazim treated plots recorded a yield increase of 33.4%.

DISCUSSION

Optimization of a ShB assay under greenhouse conditions is an important step prior to evaluation of PGPR. Our results have shown that ShB lesions are better achievable through artificial inoculation of rice seedlings with immature sclerotia of *R. solani*. The assay is reliable and easy to reproduce method for screening PGPR strains to determine their efficacy against ShB. Integral, in liquid formulation, was highly effective in suppressing ShB and in promoting rice seedling growth under greenhouse conditions. Under field conditions, Integral was also highly effective in reducing ShB severity, promoting plant height, and in increasing tillers per plant, and grain yields at a concentration of 2.20×10^9 cfu/ml when seed treatment applications were used in combination with seedling root dips and foliar spraying. These studies have shown that PGPR and bacterial endophytes play a vital role in the management of various fungal diseases. However, one of the major hurdles experienced with biocontrol agents is the lack of an appropriate delivery system. Biocontrol of rice ShB using other PGPR strains was successfully

demonstrated previously under greenhouse and field conditions (12, 43, 7, 28). Broadcast application of floating pellet formulation combined with spray application of water-soluble formulations of *B. megaterium* was found to reduce rice ShB incidence under greenhouse and field conditions (12). Multiple delivery systems of PGPR strains aimed at protecting spermosphere, rhizosphere and phyllosphere of crop plants from infection courts of pathogens was a promising means of disease management (20). Application of talc based formulation or cell suspensions of PGPR to seed, root, soil and leaves reduced rice ShB incidence with the added benefit of promoting plant growth and grain yields (21). Rabindran and Vidhyasekaran (28) reported that ShB disease could be effectively suppressed through seed treatment, soil application and foliar spraying with peat based formulation of PGPR.

Root colonization potential of PGPR also determines its field efficacy in controlling soil-borne diseases. A candidate biocontrol agent should be a potential root colonizer for successfully eliminating the pathogen in the rhizosphere. The exudates of rice roots have a significant positive effect on motility of PGPR towards roots (5). Further, *Bacillus* spp. have excellent root colonization potential. Management of rice ShB disease by Integral in the present investigation could be attributed to its application to seed and roots thereby facilitating effective root colonization and subsequent suppression of *R. solani* inoculum in the rhizosphere through competitive saprophytic ability.

Species of *Bacillus* are highly antagonistic to rice ShB pathogen (45). The fermented product of *Bacillus* strain Drt-11 reduced hyphal growth, colony diameter, and percent sclerotial germination (40-60%) of *R. solani* (19). Antibiosis mediated inhibition of ShB pathogen by *B. subtilis* was reported earlier. The *B. subtilis* strain A30 produces a thermostable and proteinase stable antibiotic (P1) that was highly effective against ShB and blast pathogens of rice (27).

Production of enzymes such as phenylalanine ammonia-lyase (PAL), peroxidase (PO), and pathogenesis-related (PR) proteins in rice leaves, and accumulation of thaumatin-like proteins, glucanases, and chitinases were the mechanisms of *R. solani* inhibition by *B. subtilis* (9). Foliar sprays with *B. megaterium* effectively reduced the percent of ShB affected tillers in rice (12). The efficacy of Integral to reduce ShB in the present study might be due to the production of siderophores, antibiotics, and lytic enzymes and induction of defense related enzymes such as PO, PAL, chitinases, β 1-3 glucanases, and phenols. Besides, direct antagonistic activity by the production of various bacterial metabolites and induction of systemic resistance by PGPR against diseases have been established as new mechanisms by which plants defend themselves against pathogen attack. Soil inoculum of *Pseudomonas fluorescens* induces disease resistance against foliar pathogens in several crops (25, 41). Any plant has endogenous defense mechanisms that can be induced by insects and pathogens. It is well known that the defense genes are inducible genes and appropriate stimuli or signals are needed for activation. Inducing the plant's own defense mechanisms by prior application of a biological inducer is thought to be a novel plant protection strategy.

Growth promoting abilities of *B. subtilis* in crop plants are well established. Rhizosphere isolates of rice produce indole-3-acetic acid (IAA), and are capable of solubilizing soil organic phosphates. They also promote seed germination, root length, plant height and dry matter production of roots and shoots (3). Inoculation of PGPR to rice fields resulted in enhanced root length (54%), root weight (74%), root volume (62%), root area (75%), shoot weight (23%), panicle emergence index (96%) and Zinc mobilization efficiency (36). *Bacillus* spp. have important plant growth promoting traits such as production of IAA, ammonia, hydrogen cyanide, siderophores, and solubilization of phosphorus besides antifungal activity (1). The culture

suspension of *B. licheniformis* CHM-1 when drenched around the roots of rice promoted seedling growth (39). Enhanced grain yields in addition to ShB control were reported with PGPR application. Prophylactic sprays with PGPR at seven days before pathogen inoculation resulted in enhanced grain yields besides reduction in ShB incidence (35). Increase in percent seed germination, root and shoot length of rice seedlings in nursery, number of tillers per plant, and ultimately grain yields in the present study by Integral might be due to the production of plant growth promoters or through indirect stimulation of nutrient uptake, and by producing siderophores or antibiotics to protect the plant from deleterious rhizosphere organisms. Production of siderophores like pseudobactin and pyoverdine which chelate the available iron in the soil, results in the death of pathogen due to lack of iron for pathogen survival. Iron deficiency in plant pathogens can cause growth inhibition, decrease in nucleic acid synthesis, inhibition of mycelial growth and sclerotial germination of *R. solani*. To conclude, the commercial formulation, Integral was highly effective at a concentration of 2.20×10^9 cfu/ml under greenhouse and field conditions as ST + SD + FS in reducing rice ShB and in promoting growth and grain yields.

PGPR are beneficial microbes that colonize rice roots effectively and enhance plant growth through a wide variety of mechanisms. PGPR have the potential to replace chemical fertilizers and pesticides in agriculture (3). However, effective control of rice ShB is feasible only when these biopesticides are used in conjunction with low rates of chemical fungicides (37). Detailed studies on mechanism of action of commercial PGPR formulations and their population dynamics in soil under submerged crop conditions are essential to formulate effective ShB management strategies at field level.

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Table 1. Sheath blight lesion development on leaf sheaths of rice by different aged sclerotia of *Rhizoctonia solani* under greenhouse conditions.

Age of Sclerotia	ShB severity ¹ (%)	Incubation period (hours) ²	Lesion size (cm ²) ³
Immature sclerotia (5-day-old)	30.6 ^a	48	1.1 ^a
Mature sclerotia (5-30-day-old)	28.7 ^a	60	0.8 ^b
Aged sclerotia (30-day-old)	29.0 ^a	60	0.7 ^b

Observations are the means of 5 replications

¹Sheath blight severity was assessed at 4 days after inoculation by Relative Lesion Height method

²Estimated as period from inoculation to appearance of 50% of water soaked lesions

³Lesion size was assessed at 4 days after inoculation by multiplying the height and width of lesions

Means followed by a common letter in the columns are not significantly different according to LSD (at $p \leq 0.05$)

Table 2. Effect of various concentrations of Integral on growth of rice seedlings and suppression of sheath blight under greenhouse conditions.

Treatment ¹	ShB severity ²	Plant height (cm) ³	Tillers /plant ⁴
Non-bacterized Control	65.5 ^a	58.3 ^d	6.3 ^d
ST+SD (2.20 x 10 ⁶ cfu/ml)	24.1 ^b	62.7 ^c	8.0 ^c
ST+SD (2.20 x 10 ⁷ cfu/ml)	20.8 ^{cd}	63.1 ^c	8.0 ^c
ST+SD (2.20 x 10 ⁸ cfu/ml)	17.9 ^d	69.3 ^b	9.5 ^b
ST+SD (2.20 x 10 ⁹ cfu/ml)	14.4 ^e	72.8 ^a	11.6 ^a
ST + SD + FS (2.20 x 10 ⁶ cfu/ml)	21.5 ^{bc}	63.3 ^c	8.0 ^c
ST + SD + FS (2.20 x 10 ⁷ cfu/ml)	18.4 ^d	63.7 ^c	8.1 ^c
ST + SD + FS (2.20 x 10 ⁸ cfu/ml)	13.5 ^e	70.8 ^{ab}	9.6 ^b
ST + SD + FS (2.20 x 10 ⁹ cfu/ml)	9.2 ^f	73.2 ^a	11.9 ^a
Carbendazim (1g/L)	7.8 ^f	58.9 ^d	7.3 ^{cd}

Values are means of five replications, two seedlings per replication

¹Strain *Bacillus subtilis* MBI 600 was applied as seed treatment (ST) before sowing, as seedling root dip (SD) on 25-days-old seedlings prior to transplanting, and foliar spray (FS) at 45 days after transplanting.

²Sheath blight severity was calculated according to Relative Lesion Height method at 52 days after transplanting

³Plant height and ⁴Tillers were taken at 60 days after transplanting

Means followed by a common letter in the columns are not significantly different according to LSD (at $p \leq 0.05$)

Table 3. Effect of various concentrations of Integral in suppression of rice sheath blight under field conditions during 2009 at A. P. Rice Research Institute, India.

Treatment ¹	%Diseased tillers/plant ²			Disease severity ³		
	Trial 1	Trial 2	Mean	Trial 1	Trial 2	Mean
ST +SD (0 cfu/ml)	95.2 ^a	92.1 ^b	93.7 ^b	65.8 ^b	56.2 ^b	61.0 ^b
ST + SD +FS (0 cfu/ml)	94.7 ^a	99.4 ^a	97.1 ^a	69.7 ^a	60.9 ^a	65.3 ^a
ST +SD (2.20 x 10 ⁸ cfu/ml)	50.3 ^b	56.1 ^c	53.2 ^c	33.7 ^c	27.5 ^c	30.6 ^c
ST +SD+FS (2.20x10 ⁸ cfu/ml)	46.3 ^c	39.7 ^d	43.0 ^d	29.4 ^d	24.5 ^d	27.0 ^c
ST +SD (2.20 x 10 ⁹ cfu/ml)	47.8 ^{bc}	37.9 ^d	42.9 ^d	31.2 ^{cd}	22.7 ^d	27.0 ^c
ST + SD +FS (2.20x10 ⁹ cfu/ml)	38.6 ^d	25.1 ^e	31.9 ^e	26.5 ^e	19.2 ^e	22.9 ^d
Carbendazim (1g/L)	37.2 ^d	20.8 ^f	29.0 ^e	19.8 ^f	16.8 ^e	18.3 ^e

Values are means of eight replications

¹Strain *Bacillus subtilis* MBI 600 was applied as seed treatment (ST) before sowing, as seedling root dip (SD) at time of transplanting (30-day-old seedlings), and foliar spray (FS) at 45 and 60 days after transplanting

²No of diseased tillers/plant were taken at 90 days after transplanting

³Sheath blight severity was calculated by Relative Lesion Height method at 90 days after transplanting

Means followed by a common letter in the columns are not significantly different according to LSD (at $p \leq 0.05$)

Table 4. Effect of various concentrations of Integral on rice growth under field conditions during 2009 at A. P. Rice Research Institute, India.

Treatment ¹	Plant height (cm) ²			No. of tillers/plant ³		
	Trial 1	Trial 2	Mean	Trial 1	Trial 2	Mean
ST +SD (0 cfu/ml)	84.5 ^d	87.8 ^c	86.2 ^d	10.0 ^d	10.3 ^c	10.2 ^c
ST + SD +FS (0 cfu/ml)	78.5 ^e	86.9 ^c	82.7 ^e	10.5 ^{cd}	10.1 ^c	10.3 ^c
ST +SD (2.20 x 10 ⁸ cfu/ml)	90.3 ^c	94.3 ^b	92.3 ^c	11.1 ^{bc}	11.6 ^b	11.4 ^b
ST +SD+FS (2.20 x 10 ⁸ cfu/ml)	91.6 ^{bc}	94.6 ^b	93.1 ^{bc}	11.4 ^b	11.8 ^b	11.6 ^b
ST +SD (2.20 x 10 ⁹ cfu/ml)	94.3 ^{ab}	97.8 ^a	96.1 ^{ab}	12.5 ^a	12.9 ^a	12.7 ^a
ST + SD +FS (2.20 x 10 ⁹ cfu/ml)	95.7 ^a	98.1 ^a	96.9 ^a	12.3 ^a	12.8 ^a	12.6 ^a
Carbendazim @1g/L	85.5 ^d	88.2 ^c	86.9 ^d	10.8 ^{bcd}	10.5 ^c	10.7 ^c

Values are means of eight replications

¹Strain *Bacillus subtilis* MBI 600 was applied as seed treatment (ST) before sowing, as seedling root dip (SD) at time of transplanting (30-day-old seedlings), and foliar spray (FS) at 45 and 60 days after transplanting

²Plant heights were taken at 90 days after transplanting

³No. of tillers/plant were taken at 60 days after transplanting

Means followed by a common letter in the columns are not significantly different according to LSD (at $p \leq 0.05$)

Table 5. Effect of various concentrations of Integral on grain yield of rice under field conditions during 2009 at A. P. Rice Research Institute, India.

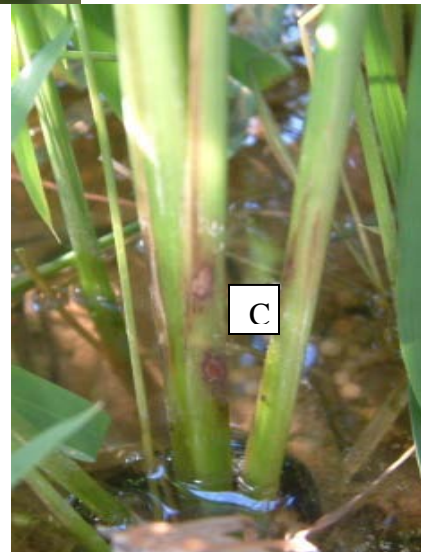
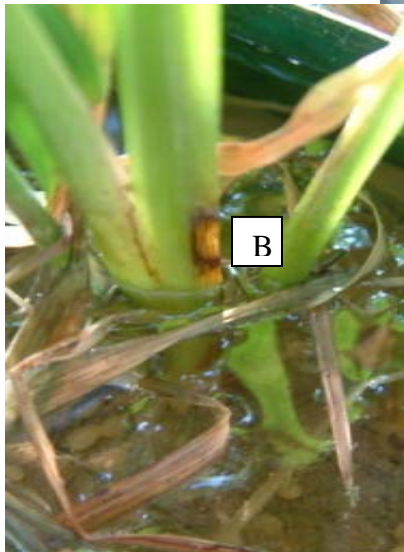
Treatment ¹	Grain yields (kg/ha) ²		
	Trial 1	Trial 2	Mean
ST +SD (0 cfu/ml)	4199 ^d	3925 ^e	4062 ^d
ST + SD +FS (0 cfu/ml)	4186 ^d	4071 ^e	4129 ^d
ST +SD (2.20 x 10 ⁸ cfu/ml)	5227 ^c	4882 ^d	5055 ^c
ST +SD+FS (2.20 x 10 ⁸ cfu/ml)	5625 ^b	5127 ^c	5376 ^b
ST +SD (2.20 x 10 ⁹ cfu/ml)	5806 ^b	5494 ^b	5650 ^b
ST + SD +FS (2.20 x 10 ⁹ cfu/ml)	6207 ^a	5922 ^a	6065 ^a
Carbendazim (1g/L)	5604 ^b	5410 ^b	5507 ^b

Values are means of eight replications

¹Strain *Bacillus subtilis* MBI 600 was applied as seed treatment (ST) before sowing, as seedling root dip (SD) at time of transplanting (30-day-old seedlings), and foliar spray (FS) at 45 and 60 days after transplanting

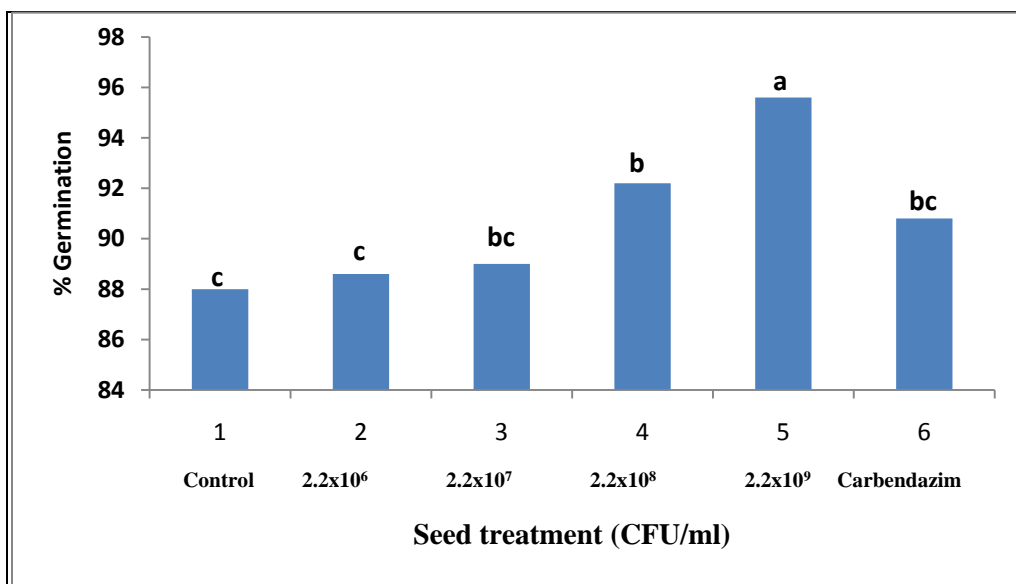
²Grain yields were taken at 90 days after transplanting

Means followed by a common letter in the columns are not significantly different according to LSD (at $p \leq 0.05$)



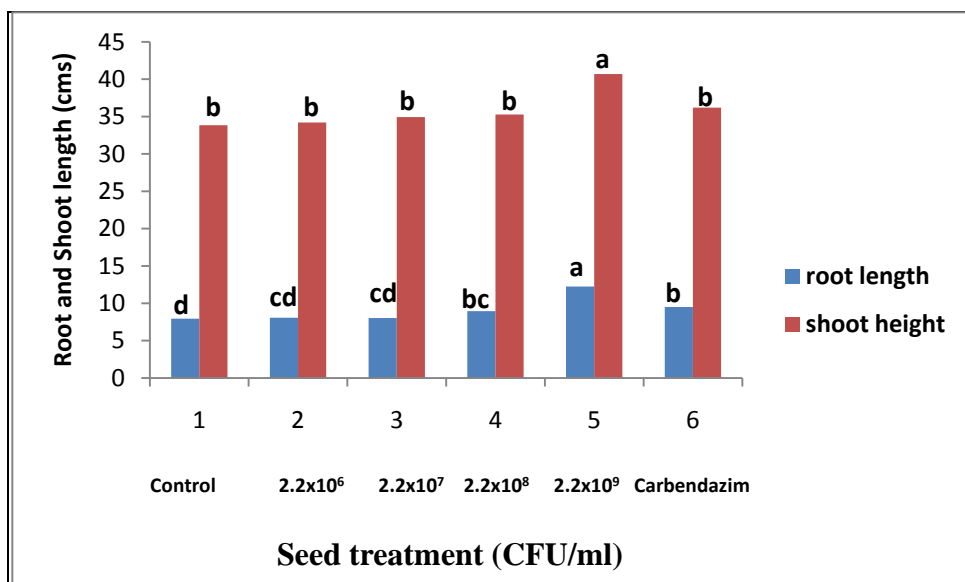
A = lesions induced by immature sclerotium; B = lesions induced by mature sclerotium and C = lesions induced by aged sclerotium

Fig. 1. Sheath blight lesions on rice leaf sheaths induced by sclerotia of different ages of *Rhizoctonia solani* under greenhouse conditions.



Integral applied as seed treatment at 2.20×10^6 , 2.20×10^7 , 2.20×10^8 , and 2.20×10^9 CFU/ml prior to seeding. Values are means of five replications, 10 seeds per replication. Means followed by a common letter are not significantly different according to LSD (at $p \leq 0.05$)

Fig. 2. Influence of various concentrations of *Bacillus subtilis* strain MBI 600 as seed treatment on seed germination of rice, CV. Swarna, at 7 days after seeding under greenhouse conditions at A. P. Rice Research Institute, India.



Integral applied as seed treatment at 2.20×10^6 , 2.20×10^7 , 2.20×10^8 , and 2.20×10^9 CFU/ml prior to seeding. Values are means of five replications, 10 seeds per replication. Means followed by a common letter are not significantly different according to LSD (at $p \leq 0.05$)

Fig. 3. Influence of various concentrations of *Bacillus subtilis* strain MBI 600 as seed treatment on seedling growth of rice, CV. Swarna at 25 days after sowing under greenhouse conditions during 2009 at A. P. Rice Research Institute, India.



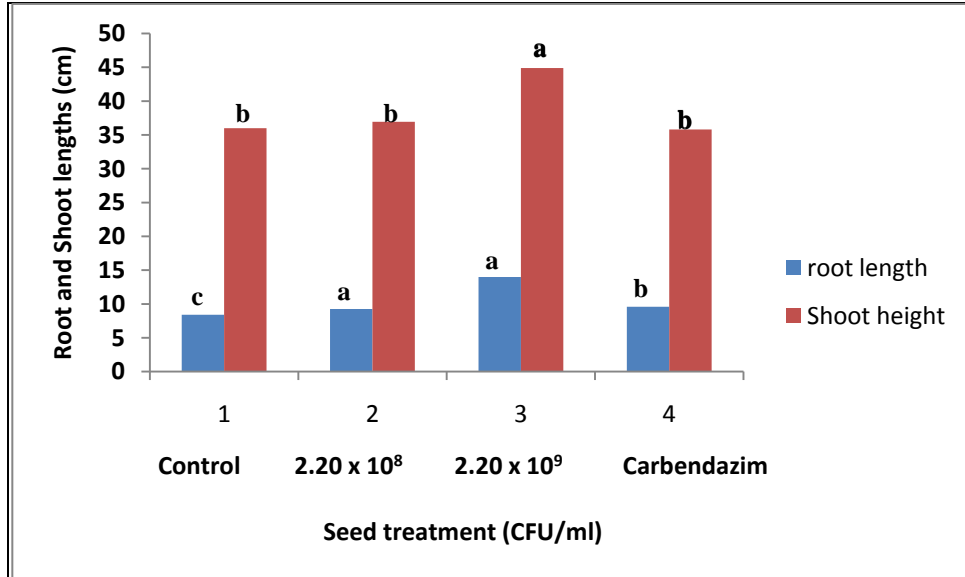
Seedlings treated with Integral



Non-bacterized control

Bacillus subtilis strain MBI 600 at 2.20×10^9 was applied as seed treatment (ST) before sowing. Seedling root dip (SD) was applied on 25-day-old seedlings prior to transplanting, and foliar spray (FS) was given at 45 days after transplanting.

Fig. 4. Effect of Integral in suppressing rice sheath blight disease under greenhouse conditions during 2009 at A. P. Rice Research Institute, India.



Integral applied as seed treatment at 2.20×10^8 and 2.20×10^9 CFU/ml prior to seeding. Values are means of four replications, 20 seeds per replication. Means followed by a common letter are not significantly different according to LSD (at $p \leq 0.05$)

Fig. 5. Influence of various concentrations of *Bacillus subtilis* strain MBI 600 as seed treatment on seedling growth of rice CV. Swarna at 30 days after sowing under field conditions during 2009 at A. P. Rice Research Institute, India.

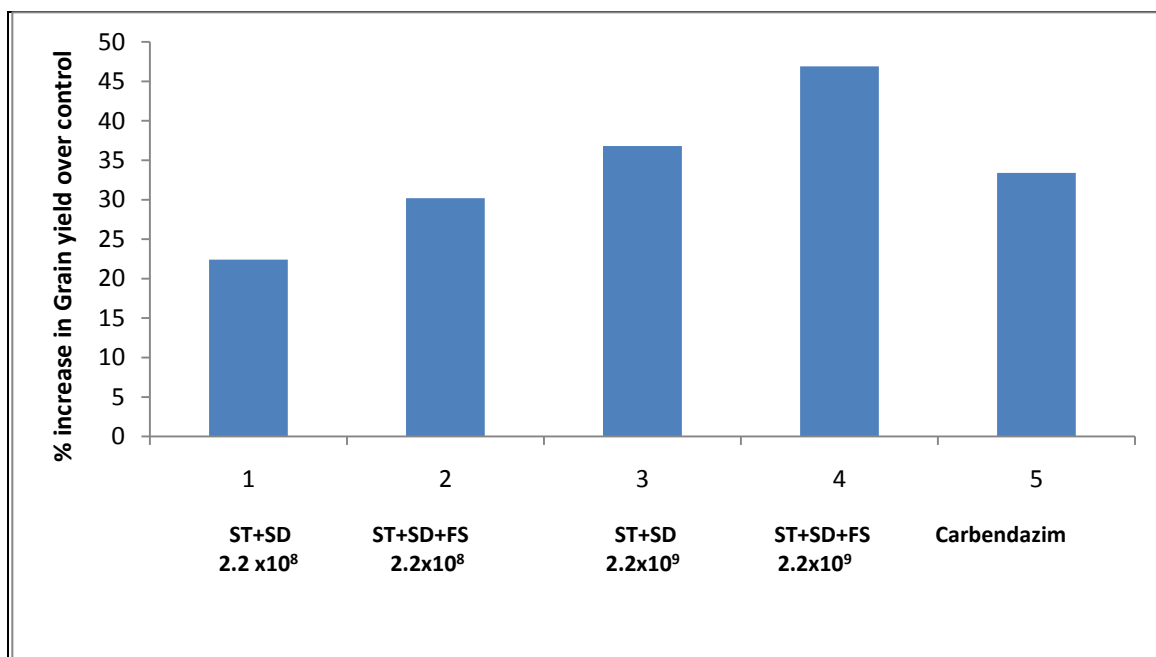


Integral treated plot

Non-bacterized control

Integral at 2.20×10^9 was applied as seed treatment (ST) before sowing. Seedling root dip (SD) was applied on 30-day-old seedlings prior to transplanting, and foliar spray (FS) was given at 45 days after transplanting.

Fig. 6. Effect of Integral in suppressing rice sheath blight severity under field conditions during 2009 at A. P. Rice Research Institute, India.



Integral applied as seed treatment (ST) prior to seeding. Seedling root dip (SD) was applied on 30-day-old seedlings prior to transplanting, and foliar spray (FS) at 45 and 60 days after transplanting. Grain yields were taken at 120 days after transplanting. Values are means of eight replications. Means followed by a common letter are not significantly different according to LSD (at $p \leq 0.05$)

Fig. 7. Influence of various concentrations of *Bacillus subtilis* strain MBI 600 on grain yield of rice CV. Swarna, under field conditions during 2009 at A. P. Rice Research Institute, India.

CHAPTER 6

MODE OF ACTION OF *BACILLUS SUBTILIS* MBI 600 IN SUPPRESSION OF RICE SHEATH BLIGHT AND ITS SEED COLONIZATION POTENTIAL

ABSTRACT

Sheath blight (ShB) of rice caused by *Rhizoctonia solani* is a major production constraint in all rice growing areas of the world. The present study focused on evaluating the mode of action of *Bacillus subtilis* strain MBI 600 (Integral®) on *R. solani* through scanning electron microscopy (SEM) and assessing its colonization potential on rice seeds. The strain MBI 600 was streaked on either side of the growing mycelia of *R. solani* in Petri dishes containing PDA and incubated for five days at 25°C. *R. solani* growing in Petri dishes not inoculated with strain MBI 600 served as control. Hyphal growth of pathogen near the zones of inhibition was processed for SEM. Integral was sprayed at 2.20×10^9 CFU/ml, onto actively growing mycelia of *R. solani* in Petri dishes containing PDA and incubated for 3 days. Interaction between strain MBI 600 and *R. solani* was studied by observing the processed mycelial bits through SEM. The effect of strain MBI 600 on *R. solani* mycelia on rice leaves was examined as follows. Detached rice leaves of 5 cm length of CV. Cocodrie from 60-days-old plants grown under GH conditions were sprayed with Integral at 2.20×10^9 CFU/ml. Later, one mature sclerotium of *R. solani* was inoculated at the center, and the leaves were incubated at 28°C for 96 h and observed. The effect of strain MBI 600 on structural integrity of sclerotia of *R. solani* was later studied by dipping sclerotia for 24 h in Integral at 2.20×10^9 CFU/ml. Sclerotia were later cut and observed in SEM. The seed

colonization potential of strain MBI 600 was determined by using rifampicin resistant mutant of MBI 600. Surface sterilized rice seeds of CV. Cocodrie were soaked in cell suspension of MBI 600 mutant at a concentration of 1×10^8 CFU/ml for 30 min. Later, seeds were divided into 8 samples and designated as Day Zero through Day Six. Seeds soaked in sterile distilled water served as control. Seeds were later dried and assessed for colonization potential by placing two rice seeds of each treatment from Day Zero to Day Six in sterile distilled water, serially diluting, and then plating onto Petri dishes containing TSA amended with rifampicin. SEM studies on antibiosis indicated that strain MBI 600 caused significant loss of structural integrity of pathogen hyphae with several deformities, shriveling, coiling, and finally lysis. Hyphae of pathogen remote from inhibition zone as well as in control plates, retained structural integrity. Integral, when sprayed on *R. solani* mycelium, resulted in colonization of MBI 600 on pathogen hyphae, maceration of tissues, shrinking and coiling of hyphae, and finally lysis. Sclerotia of *R. solani* dipped in Integral resulted in colonization of strain MBI 600, thereby causing maceration of inner sclerotial walls. Deterioration of hyphal walls was seen, finally leading to their fragmentation. Sclerotia dipped in sterile water retained structural integrity with intact inner cell walls. The population levels of strain MBI 600 were significantly decreased over time from Day Zero to Day Six after seed treatment. However, survival and colonization was evident even after six days with a population density of 0.8×10^4 CFU/seed, compared to 12×10^4 CFU/seed on Day Zero. Overall, our results suggest that Integral was highly effective in suppressing rice ShB pathogen and ShB lesion spread on detached rice leaves. Further, strain MBI 600 has good colonization potential on rice seeds, and is able to survive on seeds 6 days after application.

INTRODUCTION

Sheath blight (ShB) of rice caused by *Rhizoctonia solani* Kuhn is a common fungal disease in all rice growing countries of the world. The disease causes significant economic losses annually under high input and high production environments of Asia (20). In the United States rice growing areas of the Midsouth, ShB is the most destructive disease (4, 12, 13). The pathogen survives in the form of sclerotia for long periods in soil in the absence of hosts (15, 16). Infection of the succeeding crop is through the sclerotia that float on water (7), and these are responsible for the primary infection of ShB disease (11). Upon infection at the base of newly transplanted rice seedlings, these sclerotia produce circular to oblong grey-green, water soaked lesions (18). The pathogen later grows in the inner surface of leaf sheaths, produces infection cushions and penetrates into epidermal cells either directly or through stomata (11). Lysis of *R. solani* sclerotia and hyphae on leaf sheaths and leaf blades can lead to suppression of the disease. Unfavorable environmental conditions for vegetative growth of *R. solani* also lead to sclerotial production in soils (21). Sclerotial production of pathogen is also influenced by the antagonistic activity of soil microflora (19).

Bacteria within the group, plant growth-promoting rhizobacteria (PGPR) are widely used as biocontrol agents against rice ShB (18). These PGPR attack the pathogen and utilize the nutrients from the host hyphae. Further, the invasion of these bacterial antagonists results in lysis and death of hyphae and other survival structures of pathogen (5). Among different PGPR, *Bacillus* spp. are widely used in controlling rice ShB disease and their biocontrol potential on *R. solani* is well established. Use of these *Bacilli* as formulations to control ShB had been earlier investigated under greenhouse (10) and field conditions (17). Presently, bio-formulations of PGPR are being used for ShB management in developing countries (23, 14). Understanding the exact mechanism of action of these bacterial formulations is necessary for devising effective

management strategies for ShB at field level. Evidence on bacterial antagonism on ShB pathogen and subsequent disease suppression is an important step in determining the exact time of the application of a bioagent for breaking the disease cycle of *R. solani*. Of the different *Bacillus* spp., *B. subtilis* and *B. licheniformis* were studied extensively for their effect on growth and sclerotial formation of ShB pathogen (8). Further, these antagonists damage the surface of sclerotia and cause lysis (9). These bacterial antagonists inhibit the pathogen through mechanisms such as antibiotic production, hyperparasitism and competition for space and nutrients.

Naturally harvested rice seeds are frequently found colonized with several bacteria, of which *Bacillus* spp and *Pseudomonas* spp. are predominant. These PGPR on rice seeds exert significant antagonism on rice ShB pathogen (3). Effective root colonization is one of the key attributes of a PGPR strain for promoting plant growth and for inducing disease suppressiveness. Root exudates of rice plants were found to exert a positive influence on the motility of these bacteria towards plant roots (2). For root colonization, effective spermosphere colonization is an important prerequisite. Selective PGPR strains that are applied to rice seeds should be able to survive and colonize the root tips from the germinated seeds. However, endophytic bacteria in rice seeds can inhibit the seed colonization of the selective PGPR strains that are applied as seed treatment (1). Hence, evaluation of a PGPR strain for its colonizing ability on rice seeds is necessary for assessing its root colonization potential.

Although earlier investigations addressed the mode of action of PGPR on *R. solani*, many of them have been performed through light microscopy. However, the exact mechanism of *R. solani* inhibition by PGPR is still poorly understood. In the present study, the interaction between the commercial formulation of *B. subtilis* MBI 600 (Integral) was studied through scanning electron

microscopy (SEM). This study documented the mode of action of Integral on sheath blight sclerotia and hyphae under *in vitro* conditions and on rice leaf blades. The seed colonization potential of Integral also was determined in the present study.

MATERIALS AND METHODS

Source of rice cultivar. High yielding, conventional, long grain rice cultivar of Cocodrie, developed at Rice Research Station, LSU AgCenter, Crowley, Louisiana, USA, was obtained and used in this study. The seeds were stored at 4^o C prior to use.

Production of rice seedlings. Rice seedlings of CV. Cocodrie were grown in plastic pots containing field soil amended with Osmocote fertilizer under greenhouse conditions. Pots were initially filled with tap water and the soil was soaked completely for 72 h. Later, the soil was agitated manually to break the aggregates, and excess water was drained. Rice seedlings were produced by sowing two seeds per pot and placed on a bench in the greenhouse. Seedlings were under submerged conditions from 4th leaf stage. The pots were maintained at a temperature of 26 ± 2^oC, RH of 90%, and a photoperiod of 16 h for 60 days.

Source and production of *B. subtilis* MBI 600 in liquid formulation. The strain MBI 600 was obtained from the Phytobacteriology Laboratory strain collection, Department of Entomology and Plant Pathology, Auburn University, AL, USA. For laboratory and greenhouse studies, the liquid formulation of *B. subtilis* strain MBI 600 was produced by Becker Underwood Inc. at their fermentation facilities located in Ames, Iowa, USA. The fermented product of MBI 600 was labeled as Integral®. The product contained a minimum of 2.2 x 10¹⁰ spores/ml. The product was packaged in 500 ml bottles and shipped to Department of Entomology and Plant Pathology, Auburn University, AL, USA, to carry out studies described here.

Source of pathogen and production of sclerotia of *Rhizoctonia solani*. A multinucleate and virulent isolate of *R. solani* anastomosis group AG-1 IA was obtained from the culture collection of Dr. D. E. Groth, Rice Research Station, LSU AgCenter, Crowley, Louisiana, USA. The isolate was originally isolated from ShB infected rice seedlings. The culture was maintained on potato dextrose agar (PDA) or on rye kernels for further use. For production of sclerotia, *R. solani* was grown on PDA at $28\pm 1^{\circ}\text{C}$ in the dark conditions. The sclerotia were harvested at different time intervals and categorized according to their age as follows: immature (<5-day-old), mature (5-30 day-old) and aged (>30-days-old). The selected sclerotia were stored at 4°C prior to use.

Antibiosis of *B. subtilis* strain MBI 600 on *Rhizoctonia solani*. A loopful of strain MBI 600 stored in bottles was grown for 48 h at 25°C in 20 ml sterile tryptic soy broth (TSB) (Difco, Detroit, Michigan, USA) on a reciprocating shaker (80 rpm). Bacterial suspensions were centrifuged for 20 min at $10,000 \times g$. The resulting cell pellets were then washed two times in 0.1 M phosphate buffer (PB) (pH 6.8), resuspended in TSB amended with 20% sterile glycerol, and stored in vials at -80°C prior to use. The strain MBI 600 was retrieved from storage at -80°C , thawed and used. A loopful of inoculum was streaked onto TSA and incubated for 24 h. The *R. solani* culture was multiplied on PDA as describe above at $28\pm 1^{\circ}\text{C}$ for 36 h under dark conditions.

The antagonistic properties of strain MBI 600 were studied using SEM (5, 24). Plugs of mycelium (5 mm diameter) were cut from the edge of an actively growing fungal colony on PDA with a No. 2 cork borer, and one plug was placed in the center of each TSA plate (100 x 15 mm). Two parallel 3.5 cm long streaks of MBI 600 were then made 2 CM apart on opposite sides of the plug. The pathogen not inoculated with the selective PGPR isolate served as a control. The

plates were incubated at 25⁰C for 5 days in the dark. Fungal mycelia growing towards the inhibition zone were processed for SEM by the following procedure. Agar discs of 1mm thickness were cut from the interaction zone and placed on cover glasses (Fig 1 of Chapter 2). These were later treated with 2% osmium tetra oxide vapors for 24h at 20⁰C. The samples were later attached to aluminum stubs with double adhesive tape, coated with gold using an EMS 550X sputter coater and then imaged in a EVO50 SEM (Zeiss SMT, Inc, Germany) at 20 kV. Mycelial growth of *R. solani* in control plates was observed. The hyphal deformities near the zone of inhibition were recorded and compared with that of control plates.

Interaction between *B. subtilis* strain MBI 600 and mycelia of *Rhizoctonia solani*. The *R. solani* culture was multiplied on PDA at 28⁰C for 36 h under dark conditions. The discs of 8 mm mycelial mat of *R. solani* were sprayed with commercial formulations of strain MBI 600 at a concentration of 2.20 x 10⁹ CFU/ml and incubated for three days at 28⁰C. Fungal discs sprayed with sterile distilled served as controls. Discs of fungal mycelium were later prepared for SEM examination (25). Samples were immersed over night at 4⁰ C in 4% glutaraldehyde in 0.1M phosphate buffer, pH 7.2. The samples were washed in the same buffer, postfixed in 2% OsO₄ for 4h and dehydrated by passages through a graded aqueous ethyl alcohol series (10, 30, 50, 70, 80, 90 and 95%), then placed in 100% ethanol at room temperature for few minutes according to Tu (22). Samples were then dried in a critical-point dryer and mounted on to Al stubs with double stick adhesive tape. The samples were later coated with gold in an EMS 550X sputter coater and imaged by SEM (Zeiss EVO 50, Germany) at 20 kV. The occurrence of morphological changes in the hyphae of *R. solani* were recorded.

Observations on endospores of *B. subtilis* strain MBI 600 on rice leaves and in commercial liquid formulation. Rice seedlings of cv. Cocodrie were grown for 60 days under

GH conditions as described previously. A commercial formulation of strain MBI 600 (Integral) was sprayed at a concentration of 2.20×10^9 CFU/ml on rice seedlings at the rate of 100 ml/pot. At 24 h after spraying, the leaf blades were cut and then processed for SEM study. Leaf pieces of 5 mm length were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 90 minutes. Later, samples were dehydrated by passages through a graded aqueous ethyl alcohol series (10, 30, 50, 70, 80, 90 and 95%) and then placed in 100% ethanol at room temperature for few minutes according to Tu (22) Samples were then dried in a critical-point dryer and mounted on to Al stubs with double adhesive tape. The samples were later coated with gold in EMS 550X sputter coater and scanned by SEM (Zeiss EVO 50, Germany) at 20 kV.

The commercial liquid formulation of strain MBI 600 was mounted on to Al stubs with double adhesive tape and treated with 2% osmium tetra oxide for 24h at 20⁰ C. The specimens were later coated with gold in EMS 550X sputter coater and scanned under SEM (Zeiss EVO 50, Germany) at 20 kV.

Antagonism of *B. subtilis* strain MBI 600 on hyphae of *Rhizoctonia solani* on rice leaves.

Rice seedlings were grown under GH conditions as described previously. Leaves from 60-days-old rice seedlings were detached and brought to the laboratory in an ice box for studies on antagonism of strain MBI 600 on *R. solani*. Detached leaves of 5 cm long were sprayed with commercial formulation of strain MBI 600 at a concentration of 2.2×10^9 CFU/ml. Mature sclerotia of *R. solani* produced as described previously were inoculated individually at the center of leaves. The leaves were kept in Petri dishes containing moistened filter papers, and incubated in moist chambers at 28⁰ C for 96 h. Leaves not treated with strain MBI 600 and inoculated with sclerotia served as controls. The incubated leaves with mycelial growth of *R. solani* were cut into 5 mm long pieces and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 90 minutes.

Later, leaf pieces were dehydrated by passage through a graded aqueous ethyl alcohol series (10, 30, 50, 70, 80, 90 and 95%) and then placed in 100% ethanol at room temperature for a few minutes according to Tu (22). Samples were dried in a critical-point dryer and mounted on to Al stubs with double adhesive tape. The samples were later coated with gold in an EMS 550X sputter coater and imaged by SEM (Zeiss EVO 50, Germany) at 20 kV. Structural changes in the pathogen hyphae, frequency of penetration sites, and observations of the presence of bacterial spores on the hyphae and on leaf surfaces were recorded.

Antagonism of *B. subtilis* strain MBI 600 on sclerotia of *Rhizoctonia solani*. Mature sclerotia of *R. solani* produced as described previously were dipped in 250 ml flasks containing 100 ml of commercial formulation of strain MBI 600 at a concentration of 2.20×10^9 CFU/ml and incubated for 24 h. Sclerotia dipped in sterile distilled water served as controls. The sclerotia from different treatments were later dried on filter papers under sterile conditions at room temperatures for another 24 h. Sclerotia were cut into small pieces and placed on Al stubs with double adhesive tape. They were then treated with 2% osmium tetra oxide for 24h at 20⁰ C. Later, the specimens coated with gold in EMS 550X sputter coater and scanned under SEM (Zeiss EVO 50, Germany) at 20 kV. Changes in the structure of sclerotia were recorded.

Selection of rifampicin resistant mutants of *B. subtilis* strain MBI 600. Rifampicin resistant mutants of strain MBI 600 were selected for further evaluation of the strain's seed colonization potential. The strain MBI 600 was retrieved from storage at - 80⁰ C, thawed and used. A loopful of inoculum was streaked onto TSA and incubated for 24 h. Preliminary screening for rifampicin resistance was carried out to check the sensitivity of strain MBI 600 to 50µg/ml (50 ppm). A loopful of 24 h old culture was streaked on to Petri dishes containing TSA amended with 50 ppm of rifampicin and incubated for 48 h. The bacterial growth was completely

inhibited in rifampicin amended TSA. For developing rifampicin resistance and selection of resistant strains of MBI 600, a loopful of wild strain of MBI 600 was added to 250 ml flasks containing 100 ml of TSB amended with 100 ppm of rifampicin. Flasks were later covered with aluminum foil and incubated for 5 days on a rotary shaker at 175 rpm at room temperature ($26\pm 2^{\circ}\text{C}$). The flasks were sampled and plated for single bacterial colonies in five rifampicin amended TSA plates. The resistant colonies were selected and streaked onto TSA plates without rifampicin and incubated for 48 h. From these petri dishes, single colonies were again selected and re-streaked onto rifampicin amended TSA plates. Rifampicin marked strains of MBI 600 were obtained by repeating these steps several times. The resistant mutants were streaked on to TSA plates and compared with the wild-type for colony morphology. Mutants, which had shown marked differences with the wild type strain of MBI 600, were discarded.

Evaluation of seed colonization potential of *B. subtilis* strain MBI 600 in rice. For evaluating the seed colonization potential of MBI 600, the rifampicin resistant mutant that was comparable to the wild-type strain of MBI 600 was selected. The mutant that had similar potential with wild type strain MBI 600 in inhibiting vegetative growth, sclerotia of *R. solani*, and ShB lesion spread under controlled conditions was selected and designated as MBI 600 rif^r-1. Cell suspensions were prepared by growing MBI 600-rif^r-1 for 48 h at 25°C on TSA, harvesting in sterile distilled water, and adjusting the final concentrations at 1×10^8 CFU/ml. Rice seeds of the cv. Cocodrie were soaked in 2% sodium hypochlorite for 10 minutes and then were rinsed twice with sterile distilled water. Seeds were then soaked in cell suspension of MBI 600-rif^r-1, prepared at a concentration of 1×10^8 CFU/ml. for 30 minutes. The treated seeds were later divided into 8 samples of five seeds each. The seed samples were later dried in a laminar flow hood for 15 min and stored at room temperature. Seeds soaked in sterile distilled water

served as a control. The samples were designated as “Day Zero through Day Six” serially. The colonizing potential of strain MBI 600 on rice seeds was determined by enumerating the population levels of MBI 600-rif^r-1 on seeds in respective seed samples for every 24 h. Two seeds per each sample were taken and placed in 10 mL of sterile distilled water in test tubes and vortexed for 30 sec to resuspend the bacterial cells. Serial dilutions were prepared from the bacterial suspension and the population levels of MBI 600-rif^r-1 were enumerated by plating on TSA plates amended with 100 ppm of rifampicin. Treated seeds from Day Six sample were plated on TSA plates amended with 100 ppm of rifampicin and incubated for 48 h to check for the presence of colonies of strain MBI 600. There were seven treatments, five replications for each treatment and two seeds per replication.

Statistical analysis. All the data were analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA), and the treatment means were differentiated using PROC- GLM.

RESULTS

Antibiosis of *B. subtilis* strain MBI 600 on *Rhizoctonia solani*. SEM studies on the effect of antibiosis of strain MBI 600 revealed that sclerotial production of *R. solani* was completely inhibited near the zone of inhibition (Fig. 1a), as well as in areas remote from it (Fig. 1c). Abnormalities in the morphological structures of *R. solani* hyphae mediated by antibiosis of strain MBI 600 were clearly observed. The hyphae of ShB pathogen near the inhibition zone lost their structural integrity, leading to hyphal shrivelling. Frequent coiling of hyphae was noticed, followed by lysis (Fig. 1a). Observations did not reveal the presence of any *B. subtilis* MBI 600 endospores on the pathogen hyphae. However, in the areas remote from the inhibition zone, hyphal structures remained intact and the structural integrity was retained with initiation of

sclerotial production (Fig. 1b). Hyphae of *R. solani* in the control plates showed structural integrity. The right angled branching of hyphae was seen at regular intervals and no deformities as indicated by shrivelling of hyphal elements were noticed. The pathogen in control plates clearly produced hyphal swellings indicating the initiation of sclerotial production (Fig. 2a). Observations on mature sclerotia in control plates showed clear germination with numerous hyphal elements protruding from them (Fig. 2b).

Interaction between *B. subtilis* strain MBI 600 and mycelia of *Rhizoctonia solani*.

Studies on the interaction between *B. subtilis* MBI 600 on *R. solani* revealed complete mycoparasitism of the bacterium on *R. solani*. The bacterial growth was seen adhering to and colonizing the hyphae, thus leading to maceration of hyphal tissues (Fig. 3a). As a result, malformation of fungal structures was evident, leading to shrinking and shriveling of hyphae (Fig. 3b). The bacterial colonization over the hyphae resembled a slimy growth (Fig. 3c), and the strain MBI 600 was seen engulfing the hyphal tubes (Fig. 3d). A stress in the development of fungal mycelium was noticed due to bacterial engulfing and, as a result, deformation in hyphal filaments occurred leading to shrinking and coiling (Fig. 3e). Hyphal deterioration leading to breakage and lysis was the final step in the phenomenon of mycoparasitism (Fig. 3f). The hyphal width of *R. solani* was greatly reduced due to bacterial colonization ($3.429\mu\text{m}$) (Fig. 3g) compared to that of healthy hyphae in control plates ($5.744\ \mu\text{m}$) (Fig. 3h). Fresh colonies of strain MBI 600 were found forming on the newly developing hyphae of the test pathogen (Fig. 3i). No sclerotial production was observed in the inoculated plates. In contrast, hyphae of *R. solani* in control plates showed structural integrity with normal branching and with a normal hyphal width (Fig. 3h).

Observations on endospores of *B. subtilis* strain MBI 600 on rice leaves and in commercial liquid formulation. The SEM micrographs of liquid commercial formulation of strain MBI 600 had numerous endospores (Fig 4a) that were readily available when applied to plants. The size of endospores ranged from 1.55 to 2.06 μm in length. Endospores were also detected on rice leaf blades that possessed bumpy texture with epicuticular waxes and hairs (Fig. 4b).

Antagonism of *B. subtilis* strain MBI 600 on hyphae of *Rhizoctonia solani* on rice leaves. Numerous hyphae of *R. solani* were found protruding from the sclerotial surface on inoculated rice leaves that were not treated with strain MBI 600. These colorless hyphae completely covered the sclerotial surface and started infecting the rice leaves with numerous side branches indicating penetration sites (Fig. 5a & Fig. 5b). However, in rice leaves treated with strain MBI 600 and later inoculated with sclerotia of *R. solani*, the number of penetration sites of the pathogen was less frequent compared to control. Endospores of strain MBI 600 were seen colonizing throughout the leaf surface (Fig. 5c), resulting in fewer penetration sites of the pathogen. In the areas of penetration by the pathogen, bacterial endospores were also observed (Fig. 5d). Further, the *R. solani* hyphae showed structural abnormalities. Abnormal coiling of pathogen hyphae was noticed at regular intervals on the leaves and the hyphal tubes were found shriveled with smooth hyphal walls due to the presence of bacterial spores (Fig. 5e). On the pathogen hyphae, endospores of strain MBI 600 were seen at regular intervals (Fig. 5f), causing hyphal break down and fragmentation near the penetration sites (Fig. 5g).

Antagonism of *B. subtilis* strain MBI 600 on sclerotia of *Rhizoctonia solani*. Cross sections of mature, brown sclerotia of *R. solani* dipped in commercial formulation of strain MBI 600 have shown that the sclerotial contents were completely colonized by endospores (Fig. 6a).

These germinated endospores colonized the inner living cells of sclerotia and resulted in morphological abnormalities. Due to extensive colonization, maceration of walls of inner living cells was noticed. Cell walls of the inner/central living cells appeared smooth due to maceration and showed trends of deterioration due to germination and subsequent colonization by bacterial colonies (Fig. 6b and Fig. 6c). Maceration of cell walls and fragmentation of inner hyphal elements were observed (Fig. 6d). Cross sections of sclerotia in the control that were dipped in sterile distilled water had intact inner cell walls and the structural integrity was maintained (Fig. 6e & Fig. 6f).

Evaluation of seed colonization potential of *B. subtilis* strain MBI 600 in rice. The population of strain MBI 600 that was applied to rice seeds decreased with increase in time (Fig. 7). At Day Zero, the strain MBI 600 population/seed was 12×10^4 CFU/ml. The population levels decreased to 5.8×10^4 CFU/ml when enumerated on Day One. Significant differences were noticed between population levels of strain MBI 600/seed from Day Zero to Day One. Subsequent enumerations of populations of strain MBI 600 from Day Three through Day Six did not show any significant reductions on seeds. However, significant differences in population levels of strain MBI 600 were noticed for Day Two with Day Five and Day Six. The population levels of strain MBI 600 on rice seeds at the end of Day Six per seed were about 0.8×10^4 CFU/ml. Plating of rice seeds at the end of Day Six produced growth of MBI 600-rif^r-1 mutant on TSA plates amended with 100 ppm of rifampicin.

DISCUSSION

Our results on the mode of action of strain MBI 600 have established strong antagonism toward *R. solani* through mechanisms such as antibiosis and parasitism. Loss of structural

integrity of pathogen hyphae and reduction in sclerotial production due to antibiosis of strain MBI 600 was established. Further, the hyperparasitism of strain MBI 600 on pathogen hyphae was evident through maceration, shrinking, shrivelling, abnormal coiling and lysis of hyphal filaments. Integral, when sprayed on rice leaves, also caused abnormal coiling, shriveling and finally breaking down of pathogen hyphae due to bacterial antagonism through antibiosis and hyperparasitism. The sclerotia of *R. solani* when treated with bacterial formulation also resulted in colonization of inner sclerotial contents leading to deterioration and fragmentation. Presence of numerous endospores of the bacterium in the formulation and on rice leaves sprayed with commercial formulation indicated the potential of *B. subtilis* MBI 600 to colonize the plant surfaces, thereby leading to ShB suppression due to antibiosis, hyperparasitism and competition for space with pathogen. Isolates of *B. subtilis* cause damage to sclerotial surface and thus inhibit germination in *R. solani* (9). In addition, bacterial cells adhere to the hyphal filaments and penetrate the hyphae, thus leading to lysis and deformities in pathogens such as *Fusarium oxysporum* and *R. solani*. Sclerotia of AG-1 type are composed of three well-defined layers that include a mucilaginous surface-layer with dark brown pigmentation, an outer layer with empty cells, and an inner layer consisting of living central cells with dense contents (7). Presence of endospores in the zone of empty cell space of outer layer indicated the colonization of outer layer of empty cells that were bordered by a dark-pigmented mucilaginous surface-layer.

Our studies on seed colonization indicated that strain MBI 600 survived on rice seeds up to six days after seed treatment. Though significant reduction in population levels of strain MBI 600 was observed from Day Zero and Day Six of seed treatment. That the bacterium survived on rice seeds even after Six days indicated its colonization potential on rice seeds. Since, rice seeds treated with bacterial inoculum will normally be sown after 24 h of incubation, survival and

multiplication of bacteria will be more rapid after seed germination compared to that of ungerminated rice seeds. Furthermore, the growth of bacteria is stimulated by rice root exudates thereby facilitating their multiplication (2). Therefore, on the basis of our results, it can be concluded that *B. subtilis* strain MBI 600 is highly antagonistic to vegetative growth and sclerotia of ShB pathogen due to hyperparasitism and antibiosis. The bioagent caused deformities in both vegetative and sclerotial stages and thus suppressed ShB lesion spread on detached rice leaves. Further, the seed colonization potential of strain MBI 600 was significant in view of its survival on rice seeds up to six days. These research results confirm the role of *B. subtilis* strain MBI 600 as a potential biocontrol agent against rice ShB pathogen.

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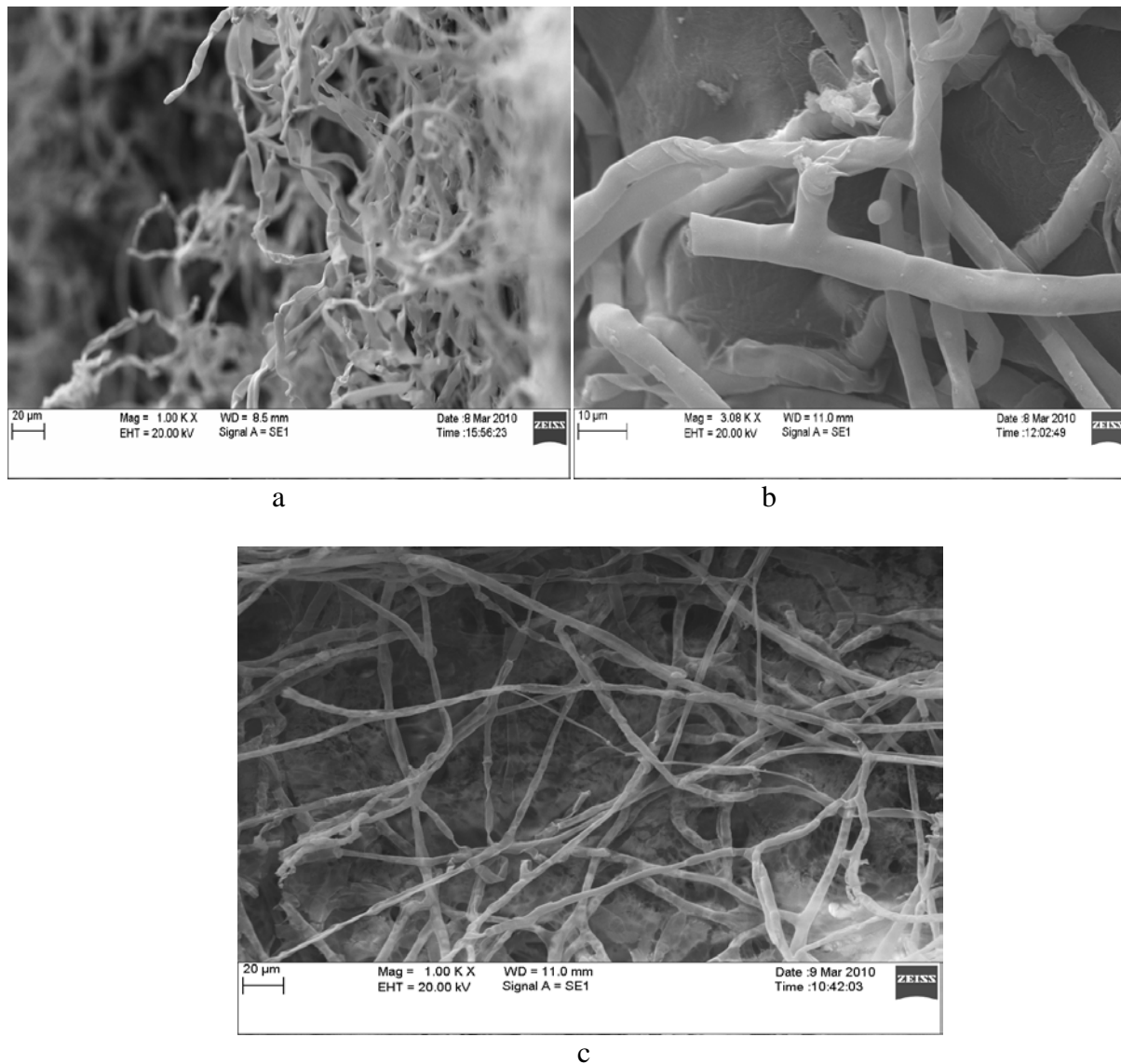
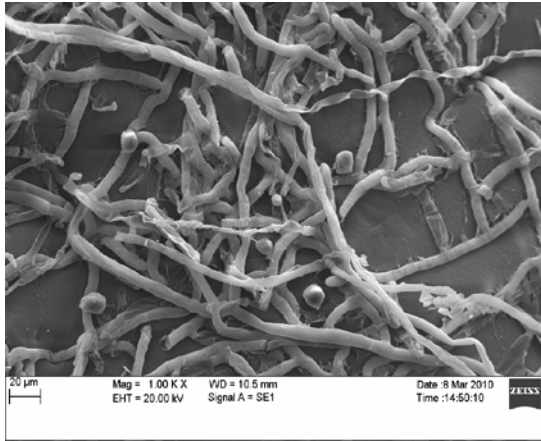
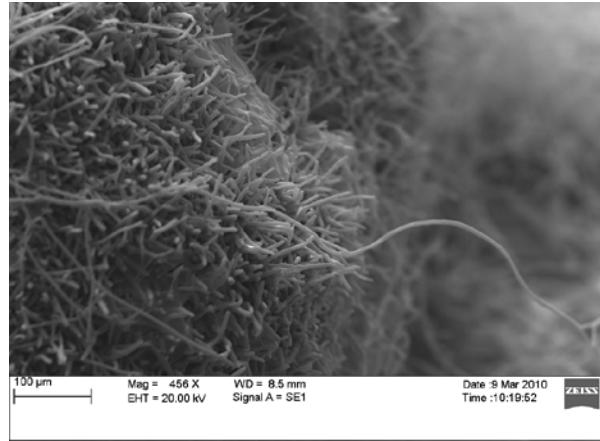


Fig. 1. Scanning electron photomicrographs showing the antibiosis mediated response of *Rhizoctonia solani* due to *Bacillus subtilis*. (a). Loss of structural integrity of test pathogen near interaction zone showing deformities with shrivelling and abnormal coiling of hyphal filaments. (b). Hyphal integrity was retained in areas remote from the interaction zone with initiation of sclerotial production. (c). Sclerotial production of pathogen away from the interaction zone was sparse and the hyphal elements show right angled branching.

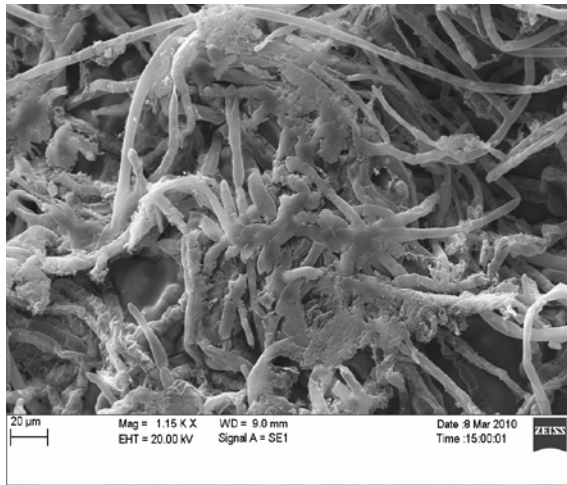


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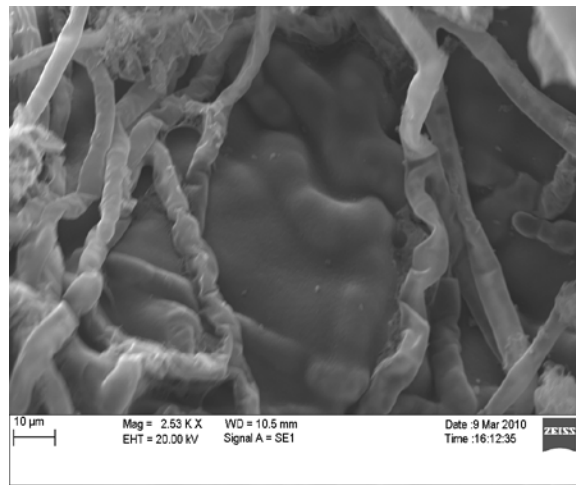


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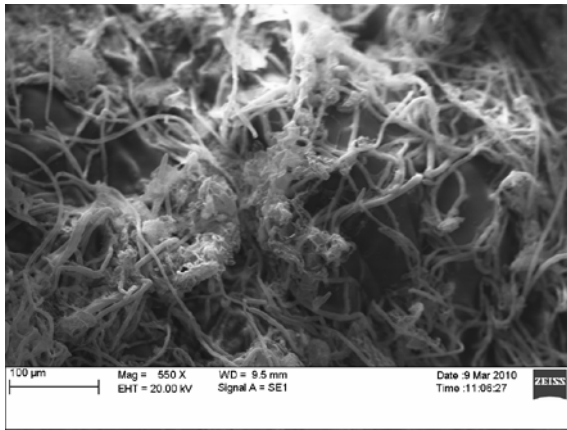
Fig. 2. Scanning electron photomicrographs of *Rhizoctonia solani* mycelia in control plates (a). Right angled branching of hyphae with initiation of sclerotia at regular intervals. (b). Mature sclerotium showing germination with budding of numerous hyphal filaments.



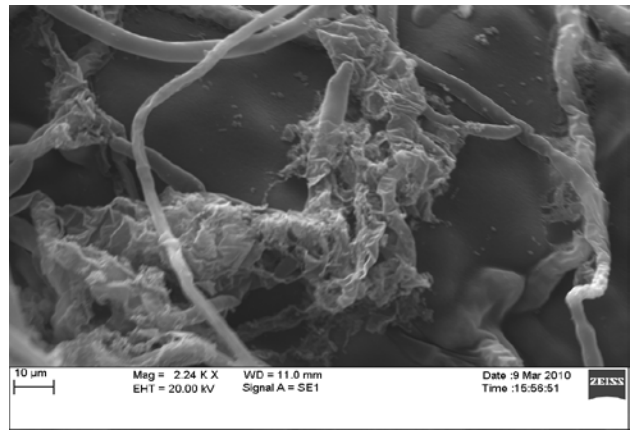
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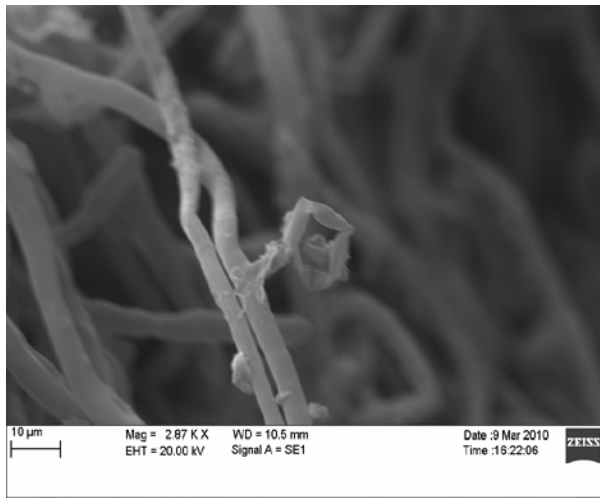
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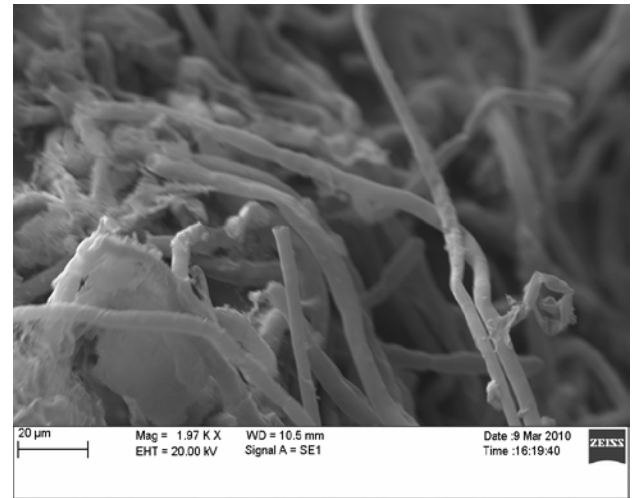
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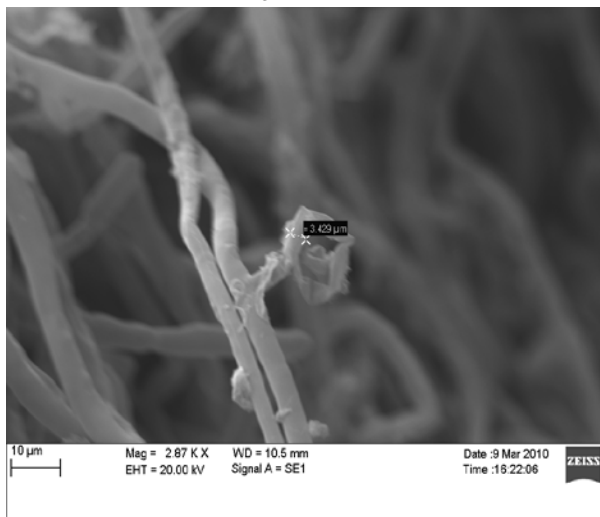
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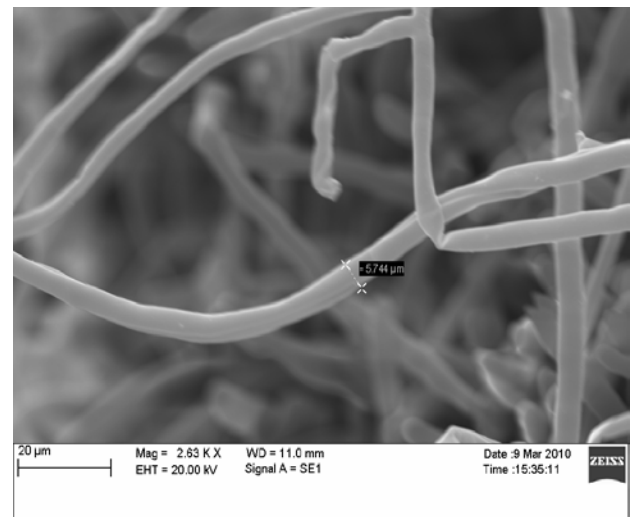
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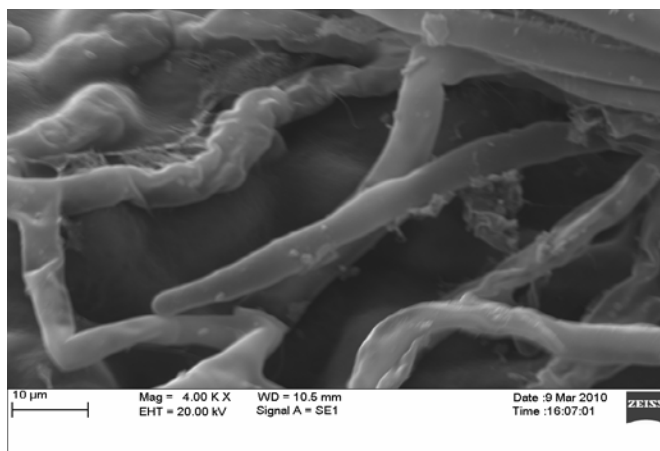
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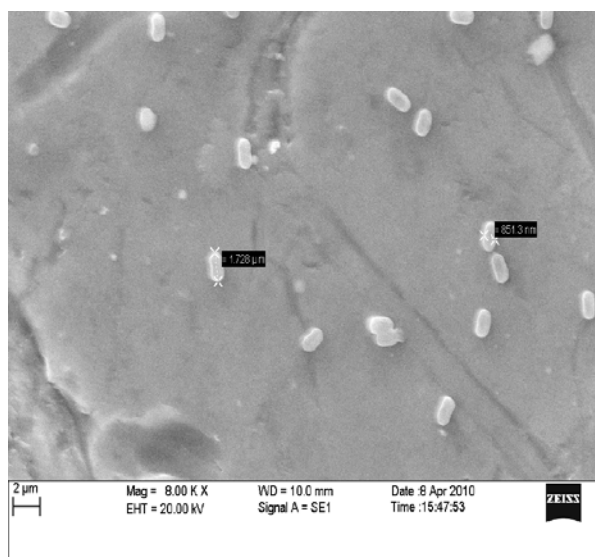


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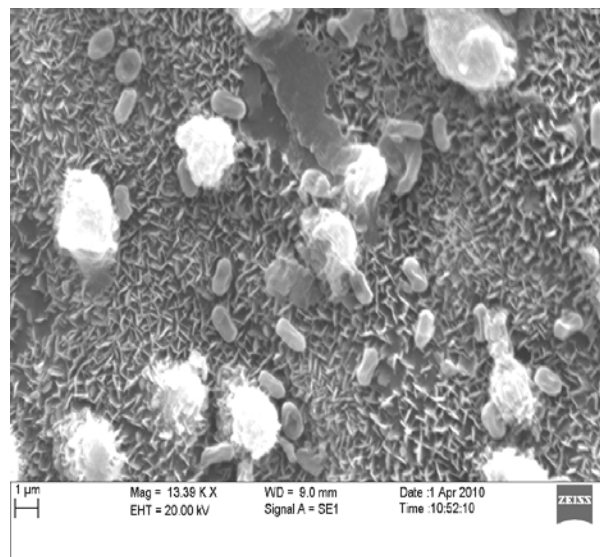


i

Fig. 3. Scanning electron photomicrographs showing the mycoparasitism of *Bacillus subtilis* MBI 600 on *Rhizoctonia solani* hyphae. (a). Maceration of hyphal tissues by the bacterium. (b). Shrinking and shriveling of pathogen hyphae. (c). Slimy growth of *B. subtilis* on pathogen hyphae. (d). Bacterial engulfing of pathogen hyphae. (e). Coiling of pathogen hyphae. (f). Hyphal lysis and breakage due to bacterium. (g). Reduced hyphal width due to mycoparasitism (h). Normal hyphal branching and width in control plates. (i). Bacterial colonies on newly developed bacterial hyphae.

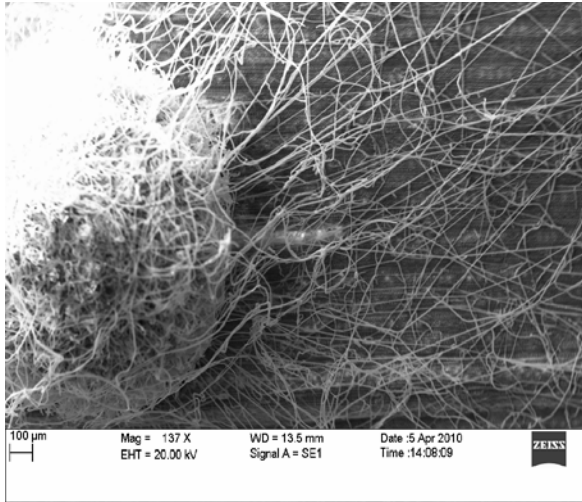


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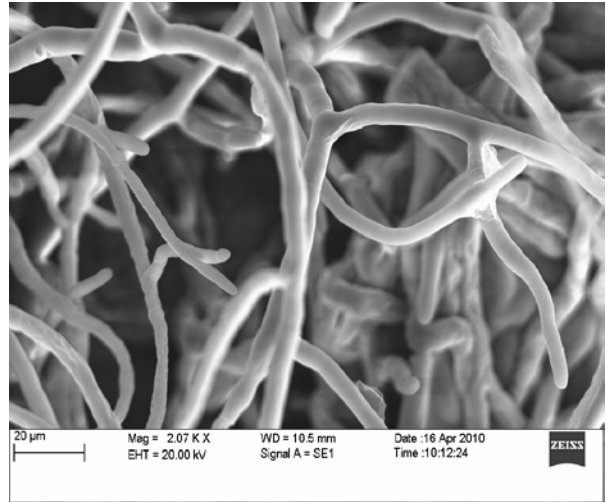


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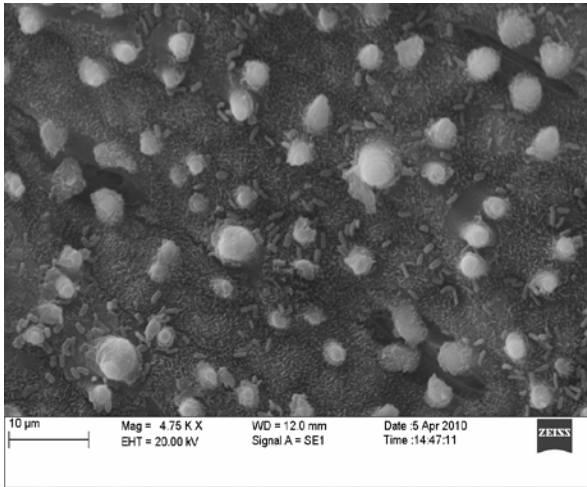
Fig.4. Scanning electron micrographs of *Bacillus subtilis* MBI 600 endospores in the formulation (Integral) (a) and on rice leaf blade (b).



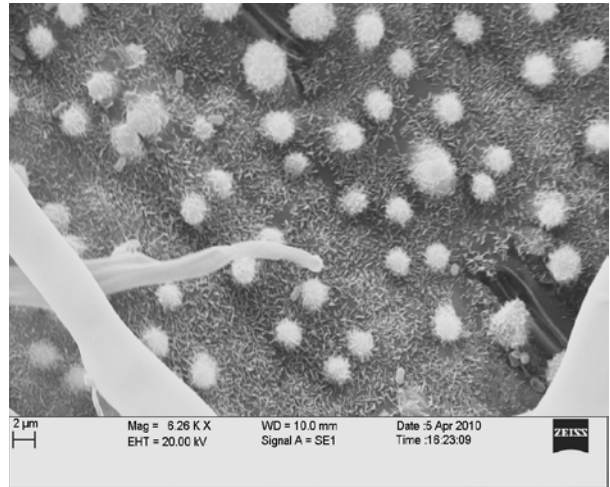
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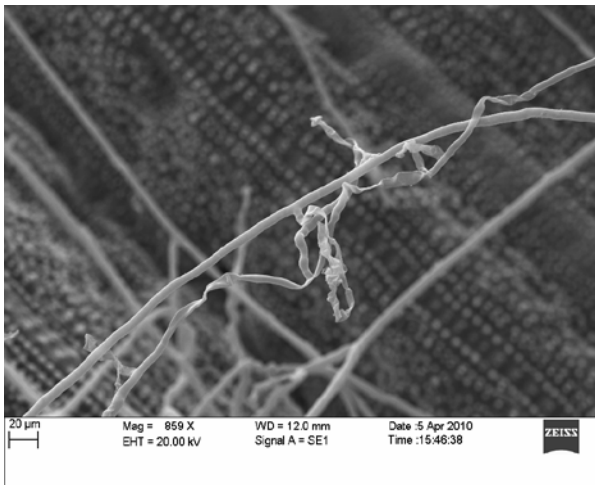
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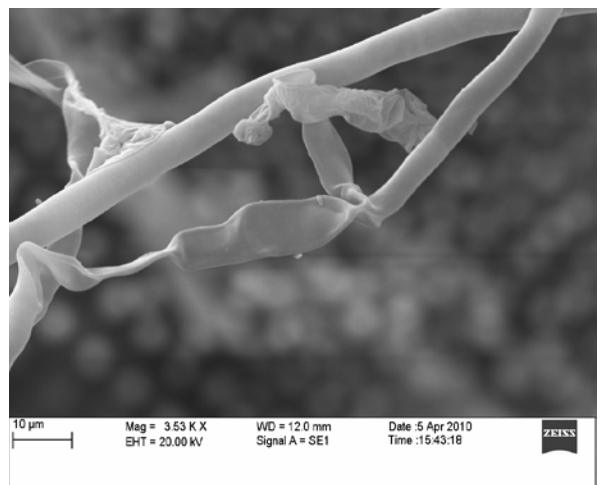
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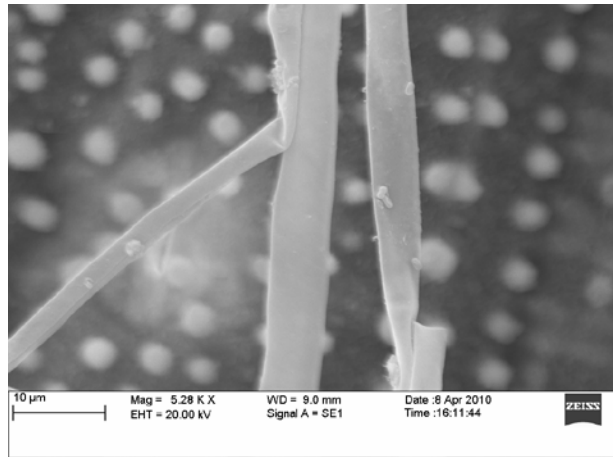
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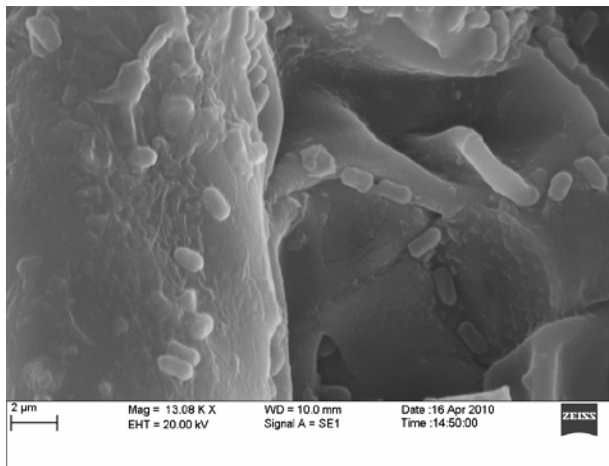


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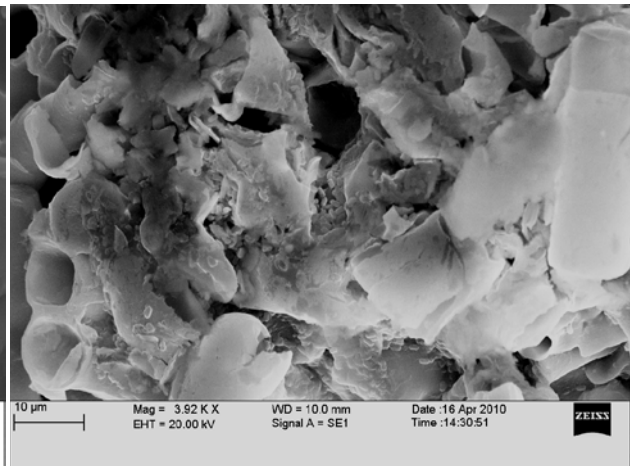


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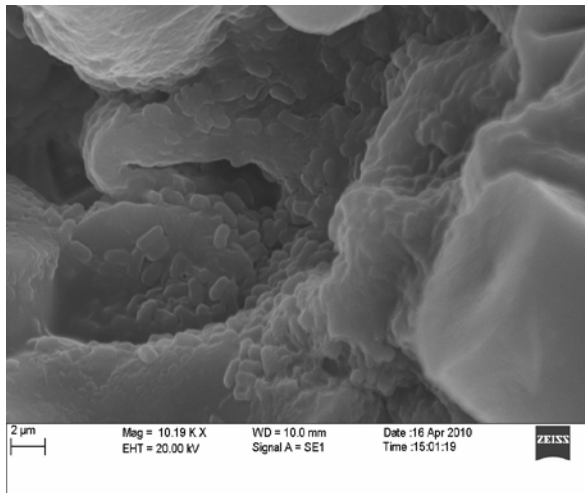
Fig. 5. Scanning electron photomicrographs showing the effect of *Bacillus subtilis* MBI 600 on *Rhizoctonia solani* on rice leaves. (a). Infection of *R. solani* from sclerotium on untreated rice leaves. (b). Production of numerous side branches by pathogen in control. (c). Colonization of bacterial endospores on rice leaves resulting in less space for pathogen infection sites. (d) Bacterial endospores in the region of pathogen penetration sites. (e). Abnormal coiling and shrivelling of pathogen hyphae on rice leaves due to bacteria. (f). Bacterial endospores on pathogen hyphae infecting rice leaf. (g). Fragmentation and hyphal break down in *R. solani* due to bacterial antagonism.



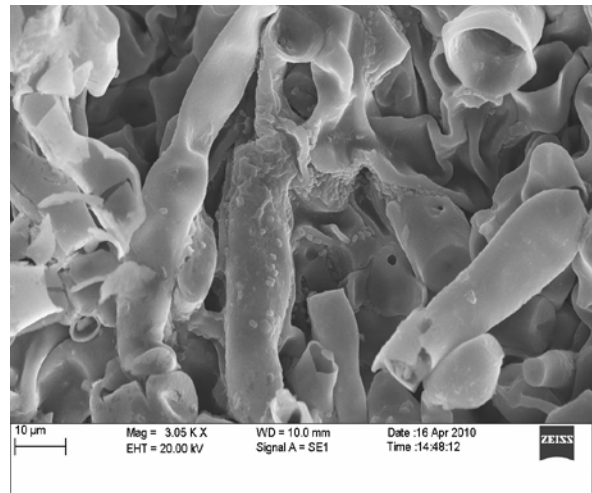
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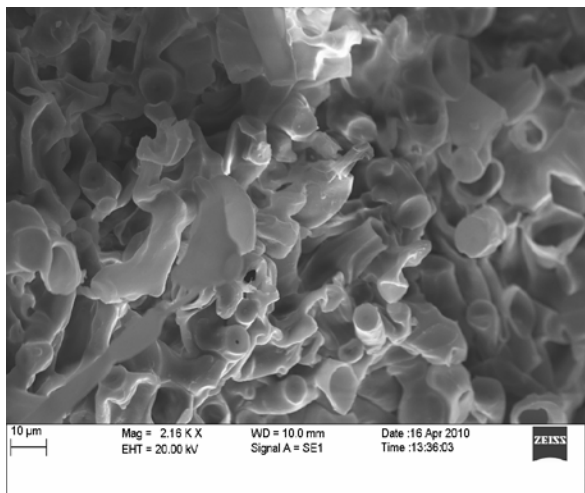
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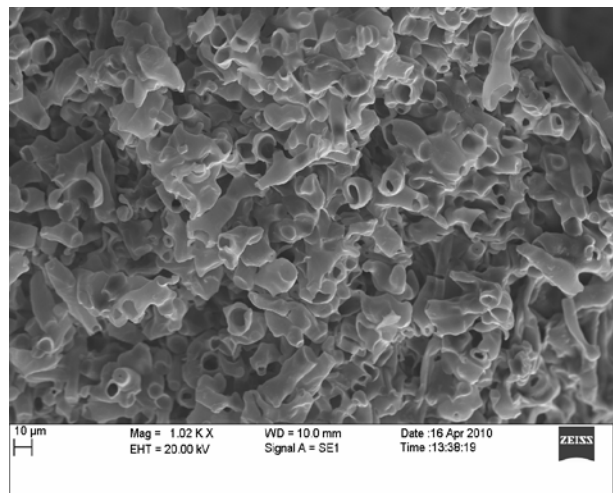
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Fig. 6. Scanning electron microscopic observations on the effect of Integral on sclerotial viability of *Rhizoctonia solani* (a). Colonization of *R. solani* sclerotial contents by endospores of *B. subtilis* MBI 600., (b). Smoothing of inner living cells of sclerotia due to maceration by bacteria., (c). Deterioration of inner living cells due to bacterial colonization and multiplication., (d). Fragmentation of inner hyphal elements of sclerotia due to bacteria, and (e & f). Healthy, untreated sclerotia with intact inner cell walls showing structural integrity