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Ultrastructural studies on the interaction between *Bacillus subtilis* MBI 600 (Integral®) and the rice sheath blight pathogen, *Rhizoctonia solani*

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The present study evaluated interactions of the biological control agent *Bacillus subtilis* strain MBI 600 (Integral®) and *Rhizoctonia solani,* the rice sheath blight pathogen, through scanning electron microscopy (SEM). Ultrastructural effects related to antibiosis were studied by dual culture of *B. subtilis* (MBI 600) and *R. solani* on potato dextrose agar (PDA) plates. Hyphal growth near the inhibition zone was processed. To study the effects of hyperparasitism on fungal ultrastructure, Integral was sprayed on *R. solani* mycelia and observations were made three days later. Interactions of *B. subtilis* (MBI 600) and *R.* solani *in planta* were determined by spraying detached rice leaves with *B. subtilis* (MBI 600) and then inoculating with sclerotia of *R. solani*. In addition, sclerotia were dipped in Integral for 24 h, and cut sections were then observed. Overall the results indicate that *B. subtilis* (MBI 600) caused loss of structural integrity, shriveling, abnormal coiling, and lysis of the *R. solani* hyphae due to antibiosis and hyperparasitism in dual culture assays. On rice leaves, *B. subtilis* (MBI 600) also caused abnormal coiling, shriveling, and break down of hyphae. Sclerotia of *R. solani* dipped in Integral resulted in colonization of *B. subtilis* (MBI 600), maceration, and fragmentation of inner walls. Our results suggest that *B. subtilis* (MBI 600) from the product Integral was highly effective in suppressing *R. solani*.

Key words: Rice, *Rhizoctonia solani, Bacillus subtilis,* SEM, hyperparasitism, antibiosis.

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 in Asia (Savary et al., 2000). In the rice growing areas of the mid-south of the United States, ShB is the most destructive disease (Groth and

Lee, 2002; Lee and Rush, 1983; Marchetti, 1983). The pathogen survives in the form of sclerotia for long periods in soil in the absence of hosts (Naiki and Ui, 1969; Palo, 1927). Infection of the succeeding crop is through sclerotia that float on water (Hashiba et al., 1972; Kozaka, 1961). Upon infection at the base of newly transplanted rice seedlings, sclerotia produce circular to oblong grey-green, water-soaked lesions (Rabindran and Vidhyasekaran, 1996). The pathogen later grows in the inner surface of leaf sheaths, produces infection cushions, and penetrates into epidermal cells either directly or through stomata (Kozaka, 1961). Unfavorable environmental conditions for vegetative growth of *R. solani* also lead to sclerotial production in soils (Townsend, 1957). Sclerotial production of pathogen is also influenced by the antagonistic activity of soil microflora (Sanford, 1956).

Some bacteria, including plant growth-promoting rhizobacteria (PGPR), have been used as biocontrol agents against rice ShB (Rabindran and Vidhyasekaran, 1996). 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 (Gupta et al., 2001). 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 was previously investigated under greenhouse (Kanjanamaneesathian et al., 1998) and field conditions (Pengnoo et al., 2000).

Presently, bio-formulations of PGPR are being used for ShB management in some developing countries (Vasudevan et al., 2002; Mew et al., 2004). Understanding the exact mechanism of action of these bacterial formulations is necessary for devising effective management strategies for ShB in the field. Evidence of 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*. Among the different species of *Bacillus*, *B. subtilis* and *B. licheniformis* have been studied extensively for their effect on growth and sclerotial formation of ShB pathogen (Henis and Inbar, 1968). Further, these antagonists damage the surface of sclerotia and cause lysis (Kanjanamaneesathian et al., 1996). These bacterial antagonists inhibit the pathogen through mechanisms such as antibiotic production, hyperparasitism, and competition for space and nutrients.

Although earlier investigations addressed the mode of action of PGPR, 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) and *R. solani* was studied through scanning electron microscopy (SEM) This study documents the mode of action of Integral on sheath blight sclerotia and hyphae under *in vitro* conditions and *in planta* on rice leaf blades.

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°C prior to use.

Production of rice seedlings

Rice seedlings of CV. Cocodrie were grown in plastic pots containing field soil amended with slow release 14-14-14 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, RH of 90% and a photoperiod of 16 h for 60 days.

Sources of *B. subtilis* MBI 600

Strain MBI 600 was provided by Becker Underwood, Ames, Iowa, USA, and was maintained at -80°C. In addition a liquid commercial formulation of MBI 600 “Integral®”, developed through fermentation process, was also provided by Becker Underwood and used in this study. The active ingredient is *B. subtilis* MBI 600 (0.18%) and with other ingredients (99.82%). The liquid commercial formulation contained a minimum of $2.2x10^{10}$ spores/ml. The product was packaged in 500 ml bottles and shipped to the 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±1°C under dark conditions. The sclerotia were harvested at different time intervals and categorized according to their age as follows: immature (<5-day-old), mature (5 to 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*

Strain MBI 600 was maintained in tryptic soy broth with 20%

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glycerol at - 80°C. For use in experiments, a loopful of frozen inoculum was streaked onto tryptic soy agar (TSA) and incubated for 24 h. The *R. solani* culture was multiplied on PDA as described above at 28±1°C for 36 h under dark conditions.

The antagonistic properties of strain MBI 600 were studied using SEM (Gupta et al., 2001; Weidenborner et al., 1989). 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. After the plates were incubated at 25°C for five days in the dark, the inhibition zones were measured using ruler. Fungal mycelia growing towards the inhibition zone were processed for SEM- method A, by the following procedure. Agar discs of 1mm thickness were cut from the inhibition zone and placed on cover glasses. For the fungal control the 1mm agar discs were sampled from the leading edge. The separated discs containing mycelia were treated with 2% osmium tetra oxide vapors for 24 h at 20°C. The treated samples were attached to aluminum stubs with double adhesive tape, coated with gold using an EMS 550X sputter coater, and then imaged using an 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 28°C for 36 h under dark conditions. The 8 mm diameter plugs cut from the leading edge of *R. solani* culture were sprayed with liquid commercial formulation of strain MBI 600 at a concentration of 2.20 x 10^9 CFU/ml and incubated for three days at 28°C. Fungal discs sprayed with sterile distilled water served as the controls. Discs of fungal mycelium were later prepared for SEM-- method B examination (Ziedan et al., 2008). Samples were immersed overnight at 4°C in 4% glutaraldehyde in 0.1M phosphate buffer, pH 7.2. The samples were washed in the same buffer, postfixed in 2% OsO_4 for 4h, and dehydrated using a graded series of ethanol according to Tu (1973). After dehydration, samples were critical-point dried with liquid carbon dioxide as a transitional fluid. The dried materials were adhered onto aluminum specimen mounts 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* was 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 greenhouse conditions as described previously. The liquid commercial formulation of strain MBI 600 was sprayed at a concentration of 2.20 x 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 samples of 5 mm length were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 90 min and were later analyzed using SEM- method B. Observations on endospores of the commercial liquid formulation of strain MBI 600 samples were analyzed using SEM- method A.

Effect of *B. subtilis* strain MBI 600 *on R. solani,* using rice leaves

Rice seedlings were grown under greenhouse conditions as described previously. Leaves from 60-day-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 until runoff with liquid commercial formulation of strain MBI 600 at a concentration of 2.2 x 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 28°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 min. Later, leaf pieces were dehydrated using SEM-method B and followed with mounting according to SEM- method A. Structural changes in the pathogen hyphae, rate of hyphal branch penetration 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 liquid commercial formulation of strain MBI 600 at a concentration of 2.20 x 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 changes in the structure of sclerotia were recorded using SEM-method A.

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 (Figure 1a), and there were very few sclerotial initiations/complete inhibitions in areas remote from it (Figure 1b). 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 (Figure 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 (Figure 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

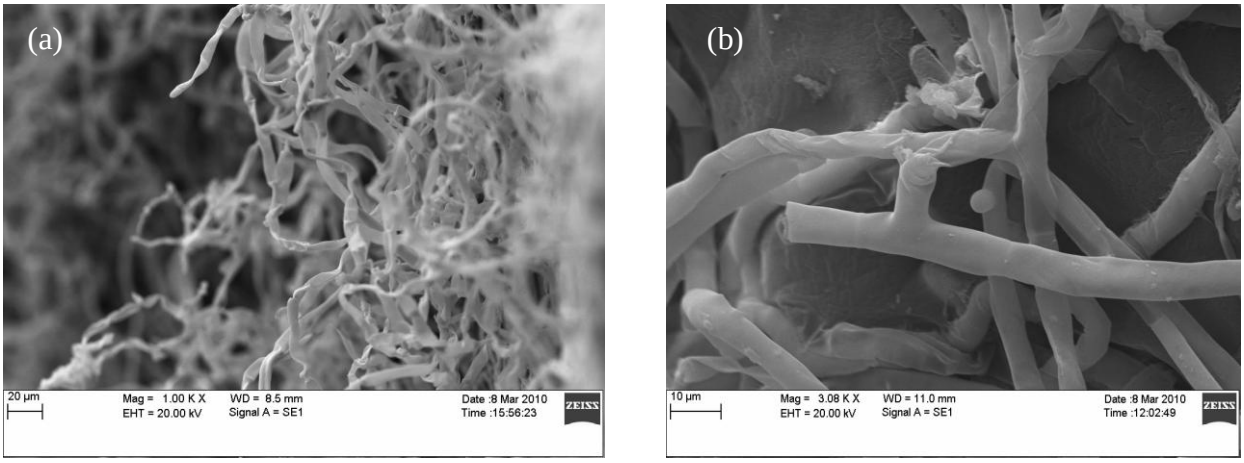

Figure 1. Scanning electron photomicrographs of antibiosis of *Rhizoctonia solani* due to *Bacillus subtilis*. (*a*). Loss of structural integrity of test pathogen near interaction zone showing deformities with shrivelling and abnormal coiling of hyphal filaments. (*b*). Hyphal integrity was retained in areas remote from the interaction zone with initiation of sclerotial production.

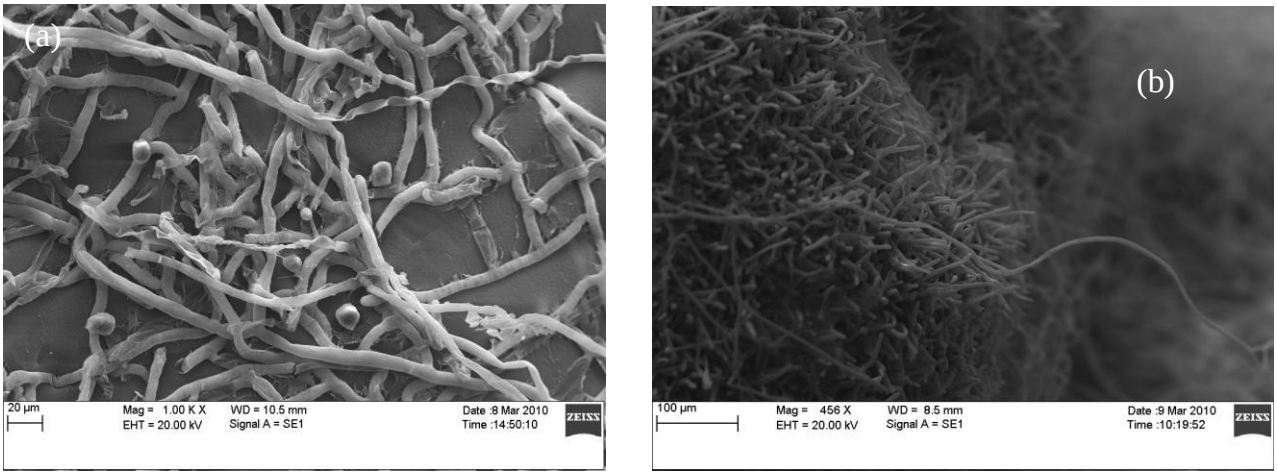

Figure 2. Scanning electron photomicrographs of *Rhizoctonia solani* mycelia in control plates without *Bacillus subtilis.* (*a*). Right angled branching of hyphae with initiation of sclerotia at regular intervals. (*b*). Mature sclerotium showing germination with budding of numerous hyphal filaments.

shrivelling of hyphal elements were noticed. The pathogen in control plates clearly produced hyphal swellings indicating the initiation of sclerotial production (Figure 2a). Observations on mature sclerotia in control plates showed clear germination with numerous hyphal elements protruding from them (Figure 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. As a result, malformation of fungal structures was evident, leading to shrinking and shriveling of hyphae (Figure 3a). The bacterial colonization over the hyphae resembled a slimy growth, and strain MBI 600 was seen engulfing the hyphae (Figure 3b). A stress in the development of fungal mycelium was noticed due to bacterial engulfing and, as a result, deformation in hyphae occurred leading to

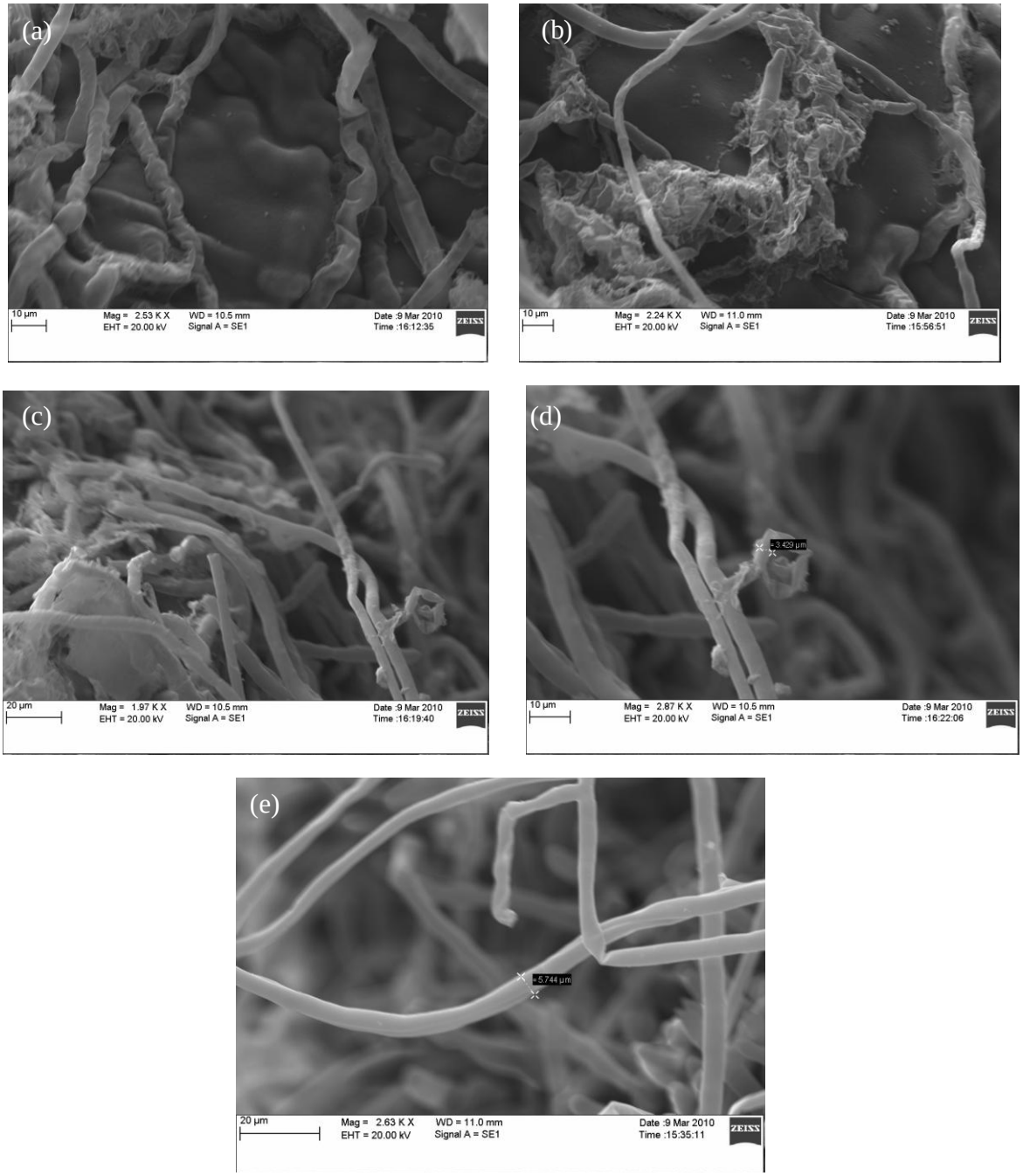

Figure 3. Scanning electron photomicrographs showing mycoparasitism of *Bacillus subtilis* MBI 600 on *Rhizoctonia solani* hyphae. (*a*). Shrinking and shriveling of pathogen hyphae. (*b*). Bacterial engulfing of pathogen hyphae. (*c*). Hyphal lysis and breakage due to bacterium. (*d*). Reduced hyphal width due to mycoparasitism. (*e*). Normal hyphal branching and width in control plates.

shrinking and coiling. Hyphal deterioration leading to breakage and lysis was the final step in the phenomenon of biological control of fungus using bacteria (Figure 3c). The hyphal width of *R. solani* was greatly reduced due to bacterial colonization (3.429µm) (Figure 3d) compared to that of healthy hyphae in control plates (5.744 µm)

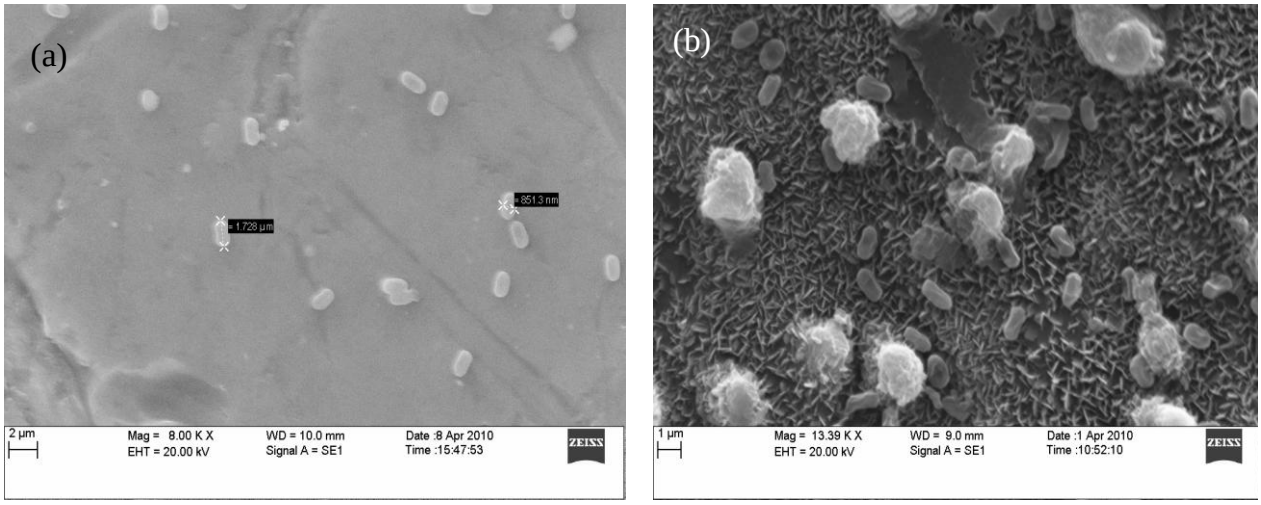

Figure 4. Scannning electron micrographs of *Bacillus subtilis* MBI 600 endospores in the commercial formulation (Integral) (*a*) and on rice leaf blade (*b*).

(Figure 3e). Colonies of strain MBI 600 were found forming on the newly developing hyphae of the test pathogen. In contrast, hyphae of *R. solani* in control plates showed structural integrity with normal branching and with a normal hyphal width (Figure 3e).

Observations on endospores of *B. subtilis strain MBI 600* on rice leaves and in commercial liquid formulation

The SEM micrographs of the liquid commercial formulation of strain MBI 600 had numerous endospores (Figure 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 after treatment with the liquid formulation. The blades exhibited a bumpy texture with epicuticular waxes and hairs (Figure 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 liquid commercial formulation strain MBI 600. These hyphae completely covered the sclerotial surface and started infecting the rice leaves with numerous side branches indicating hyphal penetration (Figure 5a and 5b). However, in rice leaves treated with strain MBI 600, and later inoculated with sclerotia of *R. solani*, the pathogen infection was less frequent compared to the control. Endospores of strain MBI 600 were seen colonizing throughout the leaf surface (Figure 5c), resulting in fewer infection sites by the pathogen. In the areas of infection by the pathogen, bacterial endospores were also observed (Figure 5d). Further, the *R. solani* hyphae showed structural abnormalities. Abnormal coiling of pathogen hyphae was noticed on the leaves and the hyphae were found shriveled due to the presence of bacterial spores (Figure 5e). On the pathogen hyphae, endospores of strain MBI 600 were seen at regular intervals (Figure 5f), causing hyphal breakdown and fragmentation near the penetration sites (Figure 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 (Figure 6a and 6b). 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. Maceration of cell walls and fragmentation of inner hyphal elements were observed (Figure 6c). Cross

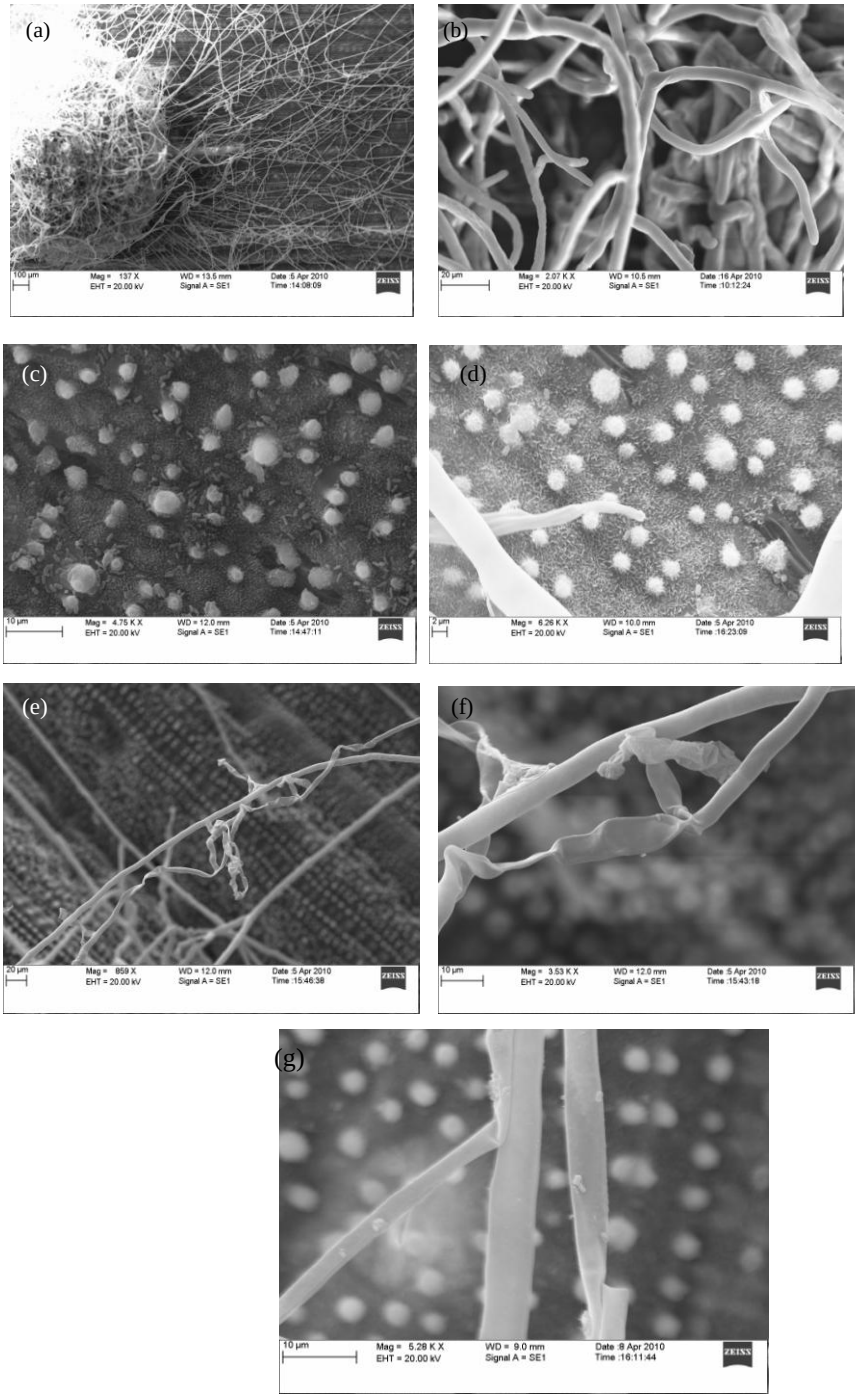

Figure 5. Scanning electron photomicrographs showing the effect of *Bacillus subtilis* MBI 600 on *Rhizoctonia solani* on rice leaves. **(a)** Infection of *R. solani* from sclerotium on untreated rice leaves. **(b)** Production of numerous side branches by pathogen in control. **(c)** Colonization of 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 breakdown in *R. solani* due to bacterial antagonism.

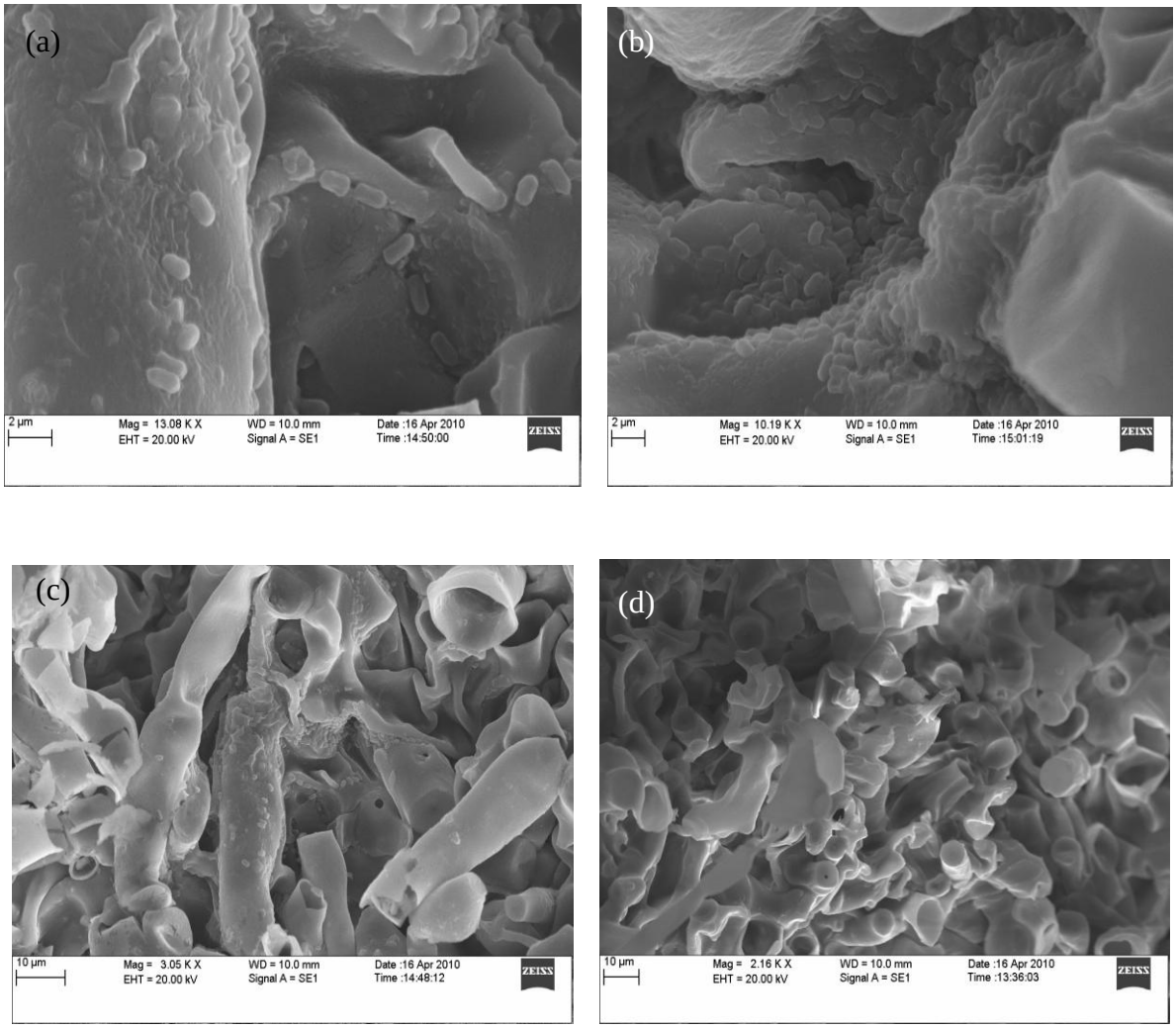

Figure 6. Scanning electron microscopic observations on the effect of Integral on sclerotial viability of *Rhizoctonia solani* (a and b). Colonization of *R. solani* sclerotial contents by endospores of *B. subtilis* MBI 600. (*c*). Fragmentation of inner hyphal elements of sclerotia due to bacteria. (*d*). Healthy, untreated sclerotia with intact inner cell walls showing structural integrity.

sections of sclerotia in the control that were dipped in sterile distilled water had intact inner cell walls, and the structural integrity was maintained (Figure 6d).

DISCUSSION

The results of ultrastructural studies presented here indicate that the mode of action of strain MBI 600 against *R. solani* involves two forms of antagonism, that is antibiosis and parasitism. Loss of structural integrity of pathogen hyphae and reduction in sclerotial production due to antibiosis of strain MBI 600 were observed. Further, hyperasitism of strain MBI 600 on fungal hyphae was evident through maceration, shrinking, shrivelling, abnormal coiling, and lysis of hyphal filaments. The commercial formulation of MBI 600, Integral, when sprayed on rice leaves, also caused abnormal coiling, shriveling, and finally breaking down of pathogen hyphae due to bacterial antagonism through antibiosis andhyperparasitism. Treatment of sclerotia of *R. solani* with Integral also resulted in bacterial colonization of inner sclerotial contents leading to deterioration and fragmentation of the fungal sclerotium. Presence of numerous endospores of the bacterium in the formulation and on rice leaves sprayed with Integral 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. In our separate studies in reducing rice sheath blight under field conditions, Integral signficantly reduced the rates of diseased tillers per plant and ShB

severity compared to control. Application of Integral as seed treatment, seedling dip and foliar spray @ 2.2 x 10^9 cfu/ml resulted in 31.9% diseased tillers (97.1% in control). Similarly, ShB severity was only 22.9% as against 65.3% in control (Vijay Krishna Kumar et al., 2012).

Our results with MBI 600 are consistent with some previous studies using different isolates of *B. subtilis.* For example, Kanjanamaneesathian et al. (1996) reported that *B. subtilis* isolates caused damage to sclerotial surface and thus inhibited outgrowth of *R. solani* hyphae. 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 *R. solani* 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 (Hashiba et al., 1972). 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.

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 bacterium caused deformities in both vegetative and sclerotial stages and thus suppressed ShB lesion spread on detached rice leaves. 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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