BIOLOGICAL CONTROL OF SCLEROTINIA SCLEROTIORUM CAUSING WHITE MOLD WITH BACILLUS AMYLOLIQUEFACIENS SUBSP. PLANTARUM

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Summary

Biological control, especially with Bacillus-based agents, offers an attractive alternative to the use of synthetic pesticides for sustainable management of white mold caused by *Sclerotinia sclerotiorum*. In this study, *S. sclerotiorum* isolates were characterized based on morphology and internal transcribed spacer sequences, followed by an assessment of their pathogenicity. Additionally, eight *Bacillus* samples were isolated from rhizospheric soil as potential biocontrol agents. Molecular analyses of 16S rDNA and gyrase subunit A (gyrA) genes combined with cultural and biochemical analyses confirmed that all isolates were Bacillus amyloliquefaciens subsp. *plantarum*. The production of hydrolytic enzymes by the isolates, and their plant growth-promoting attributes were assessed. Molecular analyses of eight antibiotic genes revealed that the genomes of all of the *Bacillus* isolates included *bacA* (bacilysin), dfnM (difficidin), fenA (fengycin), ituA (iturin), and sfp (surfactin) genes. The Bacillus isolates inhibited mycelial growth and suppressed the formation of sclerotia in an in vitro test. Treatment with the Bacillus isolates also resulted in mycelial deformities and cell wall lysis, abnormalities in apothecia, and ungerminated ascospores according to light microscopy and scanning electron microscopy analyses. These results suggest that the bacterial isolates are highly antagonistic against S. sclerotiorum. Additionally, seed bacterization with the Bacillus isolates protected mustard seedlings against S. sclerotiorum infection by up to 98%. In pot experiments, disease symptoms in mustard plants treated with a foliar spray containing Bacillus isolates decreased by up to 90%. Furthermore, the bacterial isolates increased mustard seed germination rates and improved seedling vigor, suggesting they have plant growth-promoting activities.

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Abbreviations

AUDPC	Area under progress curve
BLAST	Basic Local Alignment Search Tool
CFCF	Cell-free culture filtrate
CFU	Colony forming unit
CMC	Carboxymethyl cellulose
DDBJ	DNA Data Bank of Japan
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
IAA	Indole acitic acid
ITS	Internal transcribed spacer
LB	Luria-Bertani
MEGA	Molecular evolutionary genetics analysis
OD	Optical density
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
rDNA	Ribosomal deoxyribonucleic acid
SEM	Scanning electron microscopy
TSA	Tryptic soy agar
TSB	Tryptic soy broth

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Chapter I

Introduction

I-1 The fungus: Sclerotinia sclerotiorum

I-1-1 Biology of Sclerotinia sclerotiorum

Sclerotinia sclerotiorum (Lib.) de Bary is a soil-borne, necrotrophic, and cosmopolitan pathogenic fungus (Purdy, 1979) that can infect plants during all growth stages as well as during post-harvest storage (Bolton et al., 2006). The fungus forms sclerotia, which serve as infectious propagules in soil and survival structures. Sclerotia can remain viable for several years in fields even in adverse conditions such as heat, drought, and the presence of fungicides. Hyphae produced through the myceliogenic germination of sclerotia under ideal moisture conditions can infect plant tissues directly. Alternatively apothecia are produced from sclerotia through carpogenic development, which ultimately leads to the release of ascospores (Bolton et al., 2006).

I-1-2 Worldwide occurrences

Under suitable environmental conditions, *S. sclerotiorum* can cause a disease called white mold, which may also be referred to as Sclerotinia wilt, stalk rot, stem rot, or Sclerotinia head rot. This pathogenic fungus is capable of infecting more than 400 different plant species (Purdy, 1979; Boland and Hall 1994), including almost all types of crops from around the world. Crops from the families Brassicaceae, Compositae, and Solanaceae are especially susceptible to *S. sclerotiorum* infections.

I-1-3 Disease outbreaks in Bangladesh

In Bangladesh, *S. sclerotiorum* is emerging as a new phytopathogen capable of infecting various plants, including vegetables, fruits, and field crops in the northern part of the country (Dey et al. 2008, Rahman et al. 2015). In 2008, mustard became

the first crop confirmed to be infected by *S. sclerotiorum* (Hossain et al. 2008), followed by chilli, brinjal, and cabbage (Dey et al. 2008). In 2011, *S. sclerotiorum*-infected country bean (Prova et al. 2014) and marigold (Rahman et al. 2015) were identified, while infected jackfruit was detected in 2012 (Rahman et al. 2015a). The cultivation of several susceptible vegetables crops and suitable environmental conditions have helped the fungus become an emerging threat to sustainable crop production in Bangladesh, especially in the colder regions of the country.

I-2 Biological control of Sclerotinia sclerotiorum

I-2-1 Management of white mold

Crop management practices, such as using clean seeds, adjusting the sowing date, tilling the soil, and maintaining appropriate crop densities, may help reduce white mold incidence and severity. However, there are some limitations to these strategies (Steadman, 1979; Mueller et al., 2002). Cultural practices, such as crop rotations, are impractical and ineffective because of the long-term viability of sclerotia in the soil, and the wide host range of *S. sclerotiorum* (Bolton et al., 2006). The use of disease resistant cultivars remains the most economic and sustainable approach for managing any disease, but resistance to *S. sclerotiorum* is controlled by several genes and a few agronomic traits (Steadman, 1979). The efficacy of fungicides to control *S. sclerotiorum* has been inconsistent (Mueller et al., 2002), likely because it is difficult to appropriately time the application of the fungicide (according to ascospore release) and achieve suitable coverage (Steadman, 1979). Moreover, fungicides can have adverse effects on non-target organisms, and continuous use of chemicals may increase the chances the fungus will develop resistance to the fungicides (Mueller et al., 2002). Therefore, to overcome these limitations, researchers are considering

biological control strategies as suitable alternatives to chemicals to control *S. sclerotiorum* (Pérez-García et al., 2011).

I-2-2 Biological control with beneficial microbes

Over the last two decades, the biological control of plant pathogens has emerged as a viable disease control strategy, and is an alternative to chemical pesticides (Choudhary and Johri, 2009). A number of biocontrol agents effective against *S. sclerotiorum* from different genera such as *Bacillus*, *Pseudomonas*, and *Trichoderma* have been reported (e.g., Hou et al., 2006; Abdullah et al., 2008). Additionally, *Coniothyrium minitans*, which is a highly specific biocontrol agent, has been used commercially to eliminate viable *S. sclerotiorum* sclerotia (Gerlagh et al., 1999). However, it is limited by the slow germination of its pycnidiospores (Shi et al., 2004).

I-2-3 Biological control with Bacillus spp.

Bacillus spp. can form endospores, which are resilient structures capable of surviving in adverse conditions (McSpadden Gardener, 2004). The ecological traits of bacilli enable long-term survival, which is important for the commercialization of biocontrol products (Pérez-García et al., 2011). *Bacillus*-based biocontrol agents produce a range of different metabolites and antibiotic compounds to suppress various pests such as fungi, bacteria, nematodes, viruses, and insects (Kloepper et al. 2004, Choudhary and Johri, 2009). Recently, studies have revealed that *Bacillus* spp. can inhibit numerous air- and soil-borne plant diseases caused by fungi from different genera, including *Rhizoctonia* (Yu et al., 2002), *Colletotrichum* (Ashwini and Srividya, 2013), *Fusarium* (Zhao et al., 2014), and *Sclerotinia* (Hou et al., 2006). The antagonistic ability and plant growth-promoting attributes of *Bacillus* rhizobacteria may be useful in the development of efficient biocontrol strategies for sustainable cropping systems (Choudhary and Johri, 2009; Pérez-García et al., 2011). *Bacillus subtilis* and other closely related *Bacillus* spp. can control phytopathogens by producing antimicrobial metabolites and two dozen antibiotics, including surfactin, iturin, and fengycin (Arguelles-Arias et al., 2009; Stein, 2005). Moreover, surfactin lipopeptide acts as a potent bio surfactant and helps to maintain the densely packed multicellular structure of biofilms (Stein, 2005).

I-2-4 Need for biocontrol agents against Sclerotinia sclerotiorum

There is a need to develop sources of beneficial microbes in diverse global ecological niches to serve as potential *Bacillus*-based biofungicides (Pérez-García et al., 2011). The diverse activities of biocontrol agents may be suitable for sustainable agricultural production systems (Choudhary and Johri, 2009). Therefore, *Bacillus* samples were isolated from soil and analyzed for their suppressive ability against *S. sclerotiorum*. Additionally, a putative mode of action and plant growth-promoting attributes were studied.

I-3 Objectives of this study

- > Characterize S. sclerotiorum collected from different hosts
- > Isolate and characterize bacterial samples capable of inhibiting *S. sclerotiorum*
- Investigate the modes of action, including the molecular analysis of antibiotic genes, and plant growth-promoting attributes of collected strains
- Select bacterial strains for the biocontrol of white mold of mustard through *in vitro* and pot experiments

Chapter II

Isolation and characterization of

Sclerotinia sclerotiorum

II-1 Introduction

Sclerotinia sclerotiorum is one of the most non-specific, omnivorous, and necrotrophic phytopathogens (Purdy 1979). It is able to infect more than 400 different plant species (Boland and Hall 1994). *Sclerotinia sclerotiorum* is an emerging phytopathogen in Bangladesh, causing white mold on several plant species (Dey et al. 2008; Hossain et al. 2008; Rahman et al. 2015; Rahman et al. 2015a), which is consistent with the fact its host range is very broad (Bolton et al. 2006).

Considering it is a new phytopathogen in Bangladesh that has caused disease outbreaks, it is important that *S. sclerotiorum* is fully characterized, especially regarding its pathogenicity, at a molecular level. The internal transcribed spacer (ITS) region has been considered a universal DNA barcode marker for fungi (White et al. 1990).

The present study was completed to examine the morphological characteristics, ITS sequences (for molecular characterization), and pathogenicity of Bangladeshi isolates of *S. sclerotiorum*, and is important considering this fungus is threatening sustainable crop production in Bangladesh.

II-2 Materials and methods

II-2-1 Isolation of Sclerotinia sclerotiorum

Infected jackfruit and marigold plants exhibiting white mold symptoms were collected from the northern part of Bangladesh. Diseased plant parts and sclerotia were collected, and three isolates were purified from a single sclerotium or using the hyphal tip method. The identities of the isolates were confirmed based on mycelial and sclerotial characteristics. The isolates were maintained in potato dextrose agar (PDA) slants at 4 °C.

II-2-2 Morphological and physiological characterizations

Typical symptoms on infected plant parts, flowers, and fruits were considered during fungal isolations. Morphological and cultural characteristics (e.g., texture and color of mycelia; number and size of sclerotia) of fresh isolate cultures were analyzed. Mycelial characteristics were examined 96 h after inoculation, while sclerotial characteristics were recorded 15 d after inoculation.

Typical apothecia formed from sclerotia after conditioning using an alternating freeze-thaw process for 3 d, followed by 8 weeks of incubation on moist sand in a Petri dish.

II-2-3 Polymerase chain reaction amplification of the internal transcribed spacer region

The molecular characterization of 18 isolates was completed by sequencing part of the ITS region (Schoch et al. 2012), followed by phylogenetic analysis. Total DNA was extracted from each isolate using the ISOPLANT kit (Nippon Gene Co. Ltd., Toyama, Japan). Part of the ITS was amplified in a 25-µl reaction using primers ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGG) and the KOD FX Neo PCR mix (Toyobo, Osaka, Japan) following the manufacturer's instructions. The polymerase chain reaction (PCR) was completed in a Takara PCR Thermal Cycler Dice[®] Gradient machine (Takara, Ohtsu, Japan) using the following program: 94 °C for 2 min; 30 cycles of 98 °C for 10 s, 62 °C for 30 s, and 68 °C for 1

min; and 68 °C for 7 min. A 5-µl aliquot of each amplification sample was examined by 1% (w/v) agarose gel electrophoresis using $0.5 \times$ Tris-borate-EDTA buffer.

II-2-4 Phylogenetic analysis

The PCR amplified products were purified using diluted ExoSAP-IT reagent (USB, OH, USA), and then incubated at 37 °C for 60 min followed by 80 °C for 20 min. The nucleotide sequences were determined using dideoxy sequencing techniques at FASMAC (Kanagawa, Japan). Partial sequences were generated using the previously mentioned ITS4 and ITS5 primers. The ITS sequences were combined using the Bioedit Sequence Alignment editor 7.0.9, checked manually, corrected, and then analyzed using the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/) to identify the isolates. Phylogenetic analyses were completed using the MEGA 6 program, and a neighbor-joining tree was constructed using the Kimura two-parameter model. The phylogenetic tree was generated using the most homologous bacterial sequences available in the GenBank database. The Sclerotinia borealis MAFF 241367 sequence was used as a negative control (outgroup). Confidence values were assessed from 1,000 bootstrap replicates of the original data.

II-2-5 Pathogenicity

The pathogenicity of the isolates was assessed using mustard (*Sinapis alba* L.; Kibana-no-chikara, Takii Co.) as the common host. Detached mustard leaves from the same cultivar that were similar in age, size, and position were used for the *in vitro* evaluation of pathogenicity. A single leaf was detached from each 3-week-old plant to

ensure similar leaves were used. Three treated leaves were placed on a glass slide and positioned on top of water-moistened paper in a 15-cm plastic Petri dish to avoid direct contact with water. A 5-mm disc from a PDA plate containing actively growing *S. sclerotiorum* was used to inoculate each leaf in the same position. Plates were incubated in a growth chamber (Biotron, NK System, Osaka, Japan) at 22 °C and 85% relative humidity, with a 12-h photoperiod. Each experiment consisted of three replicates, with nine leaves in each replicate. The average infected area (%) on each detached leaf was recorded after an incubation for 1 to 5 d, and was calculated according to the following area under the disease progress curve (AUDPC) formula (Shaner and Finney, 1977):

$$AUDPC = \sum_{i=1}^{n} [(Y_{i+n1} + Y_i)/2] [X_{i+1} - X_i],$$

where Y_i = diseased area (%) at the ith observation, X_i = time (days) at the ith observation, and n = total number of observations.

To assess the pathogenicity of the *S. sclerotiorum* isolates on jackfruits, surfacesterilized detached healthy fruits were used. Agar blocks (1 cm²) containing mycelia from a 5-day-old *S. sclerotiorum* culture (on PDA) were placed on the rind surface. Each jackfruit was inoculated in four places. The experiment was completed with three replicates, with two fruits in each replicate. Therefore, each replicate consisted of eight inoculation sites. Control fruits were inoculated with PDA blocks with no mycelia. Inoculated and control fruits were incubated for 15 d in a growth chamber (MLR-351H, Sanyo, Japan) at 20 °C with 95% relative humidity, with a 12-h photoperiod.

II-2-6 Deposition of isolates

The isolates JBdSs1 and JBdSs2 were deposited into the National Institute of Agrobiological Sciences Genebank with accession numbers MAFF244849 and MAFF244850, respectively.

II-3 Results

II-3-1 Morphological and physiological characterizations

Disease symptoms on jackfruits began as a dark brown, water-soaked lesion that rapidly expanded on the fruit rind while rotting the internal fleshy tissue. Lesions with necrotic tissues subsequently developed patches of fluffy white mycelia, which were surrounded by a dark brown zone on the fruit surface. Numerous sclerotia were produced within the outer flesh and on the outside of the rind (Fig. 1A, B). In infected marigold plants, flower rot and flower drop symptoms were observed along with whitish mycelia (Fig. 1C, D)

The mycelial growth rate differed considerably among the isolates, and was used to classify the isolates in two groups, namely very fast growing and fast growing. All three isolates exhibited whitish mycelial growth with a smooth texture. On average, 15 to 20 sclerotia were produced per Petri dish (Fig. 2A). Sclerotia were irregular in shape, but generated typical apothecia after a conditioning step on moist sand in a Petri dish (Fig. 2B).

II-3-2 Molecular characterization

Molecular characterization of the three isolates by ITS sequencing indicated they were *Sclerotinia sclerotiorum*. The ITS sequences of the three isolates were identical to publicly available *S. sclerotiorum* sequences (e.g., GenBank JQ618848, HQ833450, and EF091809). The JBdSs1, JBdSs2, and MgRa-110 nucleotide sequences generated in this study were assigned GenBank accession numbers AB898681, AB898682, and AB937102, respectively (Fig. 3).

II-3-3 Phylogenetic analysis

Phylogenetic analysis of the isolates based on ITS sequences revealed the isolates belonged to a similar group of publicly available *S. sclerotiorum* sequences (Fig. 1).

II-3-4 Pathogenicity

All three isolates were highly pathogenic to their respective hosts and to mustard. In jackfruits, whitish fluffy mycelia developed at the inoculation site within 7 d, and mycelia covered approximately 3–5 cm of the fruit surface. After 14 d, up to 50% of the inoculated side of all jackfruits had rotted.



Fig. 1. White mold symptoms on *Sclerotinia sclerotiorum*-infected jackfruit and marigold. (A) White mold symptoms on the fruit surface. (B) Formation of sclerotia on the flesh and bulb under natural conditions. (C) Flower rot and flower drop symptoms in infected marigold plants. (D) Whitish mycelia on a rotting marigold flower under natural conditions.



Fig. 2. Sclerotinia sclerotiorum culture on potato dextrose agar and ascocarp formation. (A) Mycelial growth and formation of sclerotia on potato dextrose agar.(B) Formation of an ascocarp from sclerotia on sand.



Fig. 3. Neighbor-joining tree based on internal transcribed spacer sequences revealing the phylogenetic relationships among the *Sclerotinia sclerotiorum* isolates and related species. The three isolates from this study are indicated with a black diamond.



Fig 4. Sclerotinia sclerotiorum pathogenicity on jackfruits and mustard leaves.

(A) Inoculated fruits incubating in a growth chamber. (B) Development of white mold symptoms on an inoculated fruit under optimal environmental conditions inside a growth chamber. (C) Inoculated mustard leaves (from different plant growth stages) confirming the pathogenicity of the fungus. (D) Mustard leaves inoculated with *S. sclerotiorum* to identify aggressive isolates.

Chapter III

Isolation and characterization of

Bacillus isolates

III-1 Introduction

Beneficial plant growth-promoting rhizobacteria (PGPR) and biocontrol agents are associated with the surface of plant roots, and may increase plant yield through improved nutrient uptake, disease suppression, or phytohormone production. An important PGPR trait is their ability to effectively colonize the rhizosphere and stably interact with plant roots. To isolate PGPR and biocontrol agents, it is important that the rhizosphere containing plant roots is used. *Bacillus* spp. were isolated from the rhizosphere and screened for any potential inhibitory activities against *S. sclerotiorum*. The isolates were also characterized in terms of their putative mode of action and plant growth-promoting attributes.

III-2 Materials and methods

III-2-1 Isolation of *Bacillus* spp.

Bacteria were isolated from rhizospheric soil samples of various plants collected from different locations in Nihonmatsu, Fukushima, Japan (Table 1). Soil samples were added to sterilized water under aseptic conditions and prepared with gentle shaking for 1 h at 25 °C. Soil suspensions were serially diluted $(10^{-3} \text{ to } 10^{-5})$, and then treated at 80 °C for 10 min. A 100-µl aliquot was added to tryptic soy agar (TSA), which consisted of 3% tryptic soy broth (Becton Dickinson, Franklin Lakes, NJ, USA) and 1.5% agar.

The antifungal activities of the *Bacillus* isolates were screened using a dual-culture with *S. sclerotiorum* MAFF244850 (Rahman et al., 2015) as described by Souto et al. (2004). First, *S. sclerotiorum* MAFF244850 was grown on PDA at 22 °C. The

bacterial isolates were streaked on one side of an *S. sclerotiorum* plug on PDA. Plates that were inoculated with an *S. sclerotiorum* plug alone were used as the negative control. After an incubation for 5 d at 22 °C, the inhibition of mycelial growth was evaluated using the following scoring system (Perneel et al., 2007): hyphae growing on bacteria = 0; hyphae growing along the edge (< 0.5 cm) of the bacterial colony = 1; and a distinct inhibition zone (> 0.5 cm) around the bacterial colony = 2. Bacterial colonies that produced a distinct inhibition zone (scored as 2) were considered active producers of diffusible antagonistic compounds, and were selected for further characterization.

III-2-2 Morphological and physiological characterizations

Bacilli-like colonies were preliminarily identified based on their colony characteristics, including size, shape, color, margin, elevation, and opacity, and Gram stain results. Bacteria were also analyzed according to motility, formation of endospores, tolerance at 80 °C, and survival on TSA supplemented with 10% NaCl. All *Bacillus* isolates used in this study were routinely maintained on TSA at 30 °C. For broth cultures, bacteria were grown in tryptic soy broth using a BR-40LF Bio-Shaker rotary shaker (Taitec, Saitama, Japan) for 48–72 h at 30 °C and 150 rpm.

III-2-3 Polymerase chain reaction amplification of 16S rDNA

The bacterial isolates that inhibited *S. sclerotiorum* MAFF244850 growth during the primary screening experiment were analyzed by sequencing their 16S rDNA (Weisburg et al., 1991) and gyrase subunit A (*gyrA*) genes (Chun and Bae, 2000), followed by phylogenetic analyses. Total DNA was extracted from each isolate using

the ISOPLANT kit (Nippon Gene). Part of the 16S rDNA sequence was amplified in a 25- μ L reaction using universal primers 27F and 1492R (Table 2) and the KOD FX Neo PCR mix (Toyobo) according to the manufacturer's instructions. The PCR was completed in a Takara PCR Thermal Cycler Dice[®] Gradient machine (Takara) using the following program: 94 °C for 2 min; 30 cycles of 98 °C for 10 s, 62 °C for 30 s, and 68 °C for 1 min; and 68 °C for 7 min. The *gyrA* gene was amplified using primers p-gyrA-f and p-gyrA-r (Table 2) and the following program: 94 °C for 2 min; 30 cycles of 98 °C for 10 min. The other reaction conditions were the same as those used for the amplification of 16S rDNA. A 5- μ L aliquot of each amplification sample was analyzed by 1% agarose (w/v) gel electrophoresis using 0.5× Tris-borate-EDTA buffer.

III-2-4 Phylogenetic analysis

The PCR amplified products were purified using diluted ExoSAP-IT reagent (USB), and sequenced at FASMAC Co. Ltd. (Kanagawa, Japan) using the primers listed in Table 2. The sequences were combined using the Bioedit Sequence Alignment editor 7.0.9, checked manually, corrected, and then analyzed by the BLAST available on the NCBI website (http://blast.ncbi.nlm.nih.gov/) to identify isolates. Phylogenetic analyses were completed using the MEGA 6 program, and a neighbor-joining tree was constructed using the Kimura two-parameter model. The phylogenetic trees for 16S rDNA and *gyrA* were created using the most homologous bacterial sequences in the GenBank database, and the *Brevibacillus agri* DSM 6348^T and *Bacillus lichiniformis* KCTC 1918^T sequences as the outgroups, respectively. Confidence values were assessed from 1,000 bootstrap replicates of the original data.

III-2-5 Nucleotide sequence accession numbers

The 16S rDNA and *gyrA* sequences were deposited in the DNA Data Bank of Japan with the accession numbers AB971198–AB97120 and LC096068–LC096075, respectively.

III-3 Results

III-3-1 Morphological and physiological characterizations

Of 122 *Bacillus* isolates, 16 exhibited antifungal activity against *S. sclerotiorum* MAFF244850 (Table 1). All samples consisted of Gram-positive, endospore-forming, motile, and rod-shaped isolates. The bacterial colonies had folded and irregular shaped edges, and were resistant to high temperatures and saline conditions. Based on the antifungal activities observed during the initial screening (Table 1), eight isolates were selected for further investigation (RNB-88, 92, 98, 100, 107, 110, 113, and 117).

III-3-2 Molecular characterization

The 16S rDNA and *gyrA* sequences of the eight effective isolates indicated the bacteria were *Bacillus* spp.

III-3-3 Phylogenetic analysis

The phylogenetic tree generated based on the 16S rDNA sequences indicated the tested isolates were either *Bacillus subtilis* or *B. amyloliquefaciens*, while the *gyrA* sequences confirmed all isolates were *B. amyloliquefaciens* subsp. *plantarum* (Figs. 5 and 6).

Table 1	l . Bacillus i	solates col	lected fr	om rhizosph	eric soil samp	oles in Japan, ar	nd their
in vitro	o inhibitory	activities	against	Sclerotinia	sclerotiorum	MAFF244850	during
primary	screening u	using a dua	al-culture	e assay.			

Isolates	Host plant	Year	Antagonistic
			activity*
RNB-88	Onion (<i>Allium cepa</i>)	2013	2
RNB-90	Onion (<i>Allium cepa</i>)	2013	1
RNB-92	Tomato (Solanum lycopersicum)	2013	2
RNB-95	Potato (Solanum tuberosum)	2013	1
RNB-98	Potato (Solanum tuberosum)	2013	2
RNB-99	Tomato (Solanum lycopersicum)	2013	1
RNB-100	Tomato (Solanum lycopersicum)	2013	2
RNB-104	Mustard (Brassica rapa)	2013	1
RNB-107	Turnip (Brassica rapa var. rapa)	2013	2
RNB-110	Cabbage (Brassica oleracea)	2013	2
RNB-111	Cucumber (Cucumis sativus)	2013	1
RNB-113	Cabbage (Brassica oleracea)	2013	2
RNB-115	Turnip (Brassica rapa var. rapa)	2013	1
RNB-117	Eggplant (Solanum melongena)	2013	2
RNB-118	Lettuce (Lactuca sativa)	2014	1
RNB-119	Bean (Lablab purpureus)	2014	1

*Scores: 1, < 0.5 cm inhibition zone; 2, > 0.5 cm inhibition zone
Table 2. Primers used to sequence 16S rDNA and gyrA for bacterial isolate

 identifications

Targeted genes/	Primers	Primer sequences (5'–3')	T (°C)¶
metabolites			
16S rDNA	27F	AGAGTTTGATCTGGCTCAG	62
	341F	CCTACGGGAGGCAGCA	
	534R	ATTACCGCGGCTGCTGG	
	907R	CCGTCAATTCMTTTGAGTTT	
	968F	AACGCGAAGAACCTTAC	
	1492R	GGTTACCTTGTTACGACTT	
gyrA	p-gyrA-f	CAGTCAGGAAATGCGTACGTCCTT	59
	p-gyrA-r	CAAGGTAATGCTCCAGGCATTGCT	

[¶]Annealing temperature for PCR



Fig. 5. Phylogenetic tree of the *Bacillus* isolates (black diamond) based on 16S rDNA sequences. The tree was constructed using the neighbor-joining method, and genetic distances were generated with the Kimura two-parameter model. The branch numbers refer to bootstrap confidence percentages from 1,000 trial replications. *Brevibacillus agri* DSM 6348^T was used as an outgroup. Numbers in parentheses correspond to the accession numbers in the GenBank database. Bar: 2% sequence divergence



0.02

Fig. 6. Phylogenetic tree of the *Bacillus* isolates (black diamond) based on the *gyrA* sequences. The tree was constructed using the neighbor-joining method based on the Kimura two-parameter model for determining genetic distances. The branch numbers refer to bootstrap confidence percentages from 1,000 trial replications. *Bacillus licheniformis* KCTC 1918^T was used as an outgroup. Numbers in parentheses correspond to the accession numbers in the GenBank database. Bar: 2% sequence divergence

Chapter IV

Biological control of Sclerotinia sclerotiorum with Bacillus spp.

IV-1 Introduction

Sclerotinia sclerotiorum is pathogenic to a broad range of plant hosts worldwide (Bolton et al. 2006). It survives in the soil for several years as sclerotia, which serve as a long-term inoculum source. Under suitable environmental conditions, sclerotia can produce apothecia that release numerous ascospores. Strategies targeting either sclerotia or ascospores have been used to control the fungus.

Metabolites synthesized by beneficial bacteria, fungi, and actinomycetes are able to prevent the growth of other pathogenic microorganisms. Some bacteria can produce various metabolites and antibiotics if grown in suitable culture medium under appropriate conditions. *Bacillus* spp. are usually associated with antibiosis and eventually parasitism and competition. The objective of this study was to characterize the activities of *Bacillus* spp. during the biological control of *S. sclerotiorum*.

IV-2 Materials and methods

IV-2-1 Production of hydrolytic enzymes and plant growth-promoting compounds

Protease activity was detected based on the presence of a clear zone on a Luria-Bertani (LB) plate amended with 2% skim milk powder after an incubation at 28 °C for 48 h (Perneel et al., 2007). Extracellular chitinase activity was studied using chitin-supplemented minimal medium agar plates (0.2% colloidal chitin) (Singh et al., 1999), and β -1,3-glucanase activity was examined on minimal medium supplemented with laminarin as the sole carbon source (Dunne et al., 1997). The isolates were spotinoculated at the center of each plate. After an incubation at 30 °C for 7 d, the development of clear zones around colonies was examined. Cellulase production was determined based on the appearance of a halo around a bacterial colony on a carboxymethyl cellulose-amended Czapek-mineral salt agar plate.

Indoleacetic acid (IAA) production by the bacterial isolates was examined according to the method of Minaxi et al. (2012). Briefly, bacterial cultures were inoculated separately in LB broth containing L-tryptophan (3 mg ml⁻¹) and incubated at 28 ± 2 °C for 5 d. Cultures were centrifuged at 3,000 rpm for 30 min, and a 2-ml aliquot of the supernatant was mixed with two drops of orthophosphoric acid and 4 ml Salkowski's reagent (50 ml 35% perchloric acid, 1 ml 0.5 M FeCl₃). Indoleacetic acid production was indicated if the solution turned pink. Bacterial isolates were also tested for siderophore production on chrome-azurol S medium (Alexander and Zuberer, 1991). Bacterial isolates were used to spot-inoculate the solid medium, which was then incubated at 30 °C for 48–72 h. The formation of an orange to yellow halo around the colonies was considered to indicate siderophore production.

Phosphate solubilization on solid medium was detected by adding individual isolates on Pikovskaya's agar plates containing tri-calcium phosphate (Kumar et al., 2012). The plates were incubated at 30 °C for 4–5 d. The development of a clear halo around colonies indicated the isolates were capable of solubilizing phosphate. Additionally, bacterial isolates were screened for the production of ammonia using peptone water broth (Ahmad et al., 2008). Bacterial cultures were inoculated in 10 ml peptone water and incubated at 30 °C for 4 d. The accumulation of ammonia was detected if the solution became brown to yellow following the addition of 0.5 ml Nessler's reagent.

IV-2-2 Biofilm formation

Biofilm formation by *Bacillus* isolates was monitored using a microtiter plate assay according to the method described by Jackson et al. (2002). Briefly, LB medium was

inoculated with diluted bacterial cells at the mid-exponential growth stage. The inoculated medium was aliquoted into each well of a 96-well cell culture plate, which was then incubated at 28 °C. The medium was discarded after 24, 48, 72, and 96 h, and the plate was washed with water and then stained with 1% crystal violet. The plates were then rinsed with water, and the dye was solubilized with 33% acetic acid. Biofilm formation was quantified by measuring the optical density at 630 nm (OD_{630}) of each well using a Model 680 microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

IV-2-3 Detection of antibiotic genes using the polymerase chain reaction

Putative genes related to the biosynthesis of antifungal compounds, including surfactin, iturin, fengycin, bacilysin, and difficidin, were examined by PCR. Total DNA was purified as described above and diluted 100-fold in sterile distilled water. Each gene was PCR amplified using the KOD FX Neo PCR mix (Toyobo) following the manufacturer's instructions. Genes for surfactin (lipopeptide antibiotic), bacilysin (dipeptide antibiotic), bacillibactin (iron-siderophore), bacillaene (polyketide antibiotic), and difficidin have been detected in the genome of *Bacillus* isolates (Chen et al., 2009a; Stein, 2005). Therefore, eight primer pairs (Table 3) were used to amplify the putative antibiotic genes *bacA*, *beaB*, *dfnM*, *dhbF*, *fenA*, *ituA*, *sfp*, and *srf* (Arguelles-Arias, 2009; Zhao et al., 2014). The following PCR program was used: 94 °C for 2 min; 30 cycles of 94 °C for 30 s, annealing temperature indicated in Table 1 for 30 s, and 72 °C for 1 min; and 72 °C for 10 min. After analyses by agarose gel electrophoresis, the PCR products were purified using the ExoSAP-IT kit. The purified amplicons were sequenced at FASMAC. The resulting sequences were

compared with those of similar genes from Bacillaceae bacteria. Phylogenetic trees were constructed using the neighbor-joining method as described previously.

IV-2-4 In vitro inhibition of Sclerotinia sclerotiorum

Bacterial isolates were tested for their ability to suppress *S. sclerotiorum* growth using an *in vitro* dual-culture method (Souto et al., 2004). A 0.5-cm plug containing mycelia taken from a 3-day-old *S. sclerotiorum* MAFF244850 culture was placed on one side of a PDA plate. A single bacterial colony was streaked in a straight line at a distance of about 4 cm from the fungal plug. Plates without a bacterial isolate served as controls.

The antifungal activities of bacterial cell-free culture filtrates (CFCFs) were also evaluated. The CFCF of each isolate was obtained from bacterial cultures (log-phase) by centrifugation at 8,000 rpm for 10 min at 4°C, and subsequent filtration with a 0.20-µm DISMIC-25CS membrane filter (Advantec, Toyo Roshi Kaisha Ltd., Tokyo, Japan). A plug containing *S. sclerotiorum* MAFF244850 mycelia was prepared as described above, and placed in the center of a PDA plate. The bacterial CFCF was aliquoted into wells (0.5 cm) made on four sides of each plate, 3 cm from the fungal plug. Plates without the CFCF served as controls.

The inhibitory effects of *Bacillus* volatile compounds on *S. sclerotiorum* growth were also analyzed. A 2-day-old culture of each bacterial isolate was streaked on a TSA plate, and incubated for 24 h at 28 °C. A PDA plate with actively growing *S. sclerotiorum* MAFF244850 was prepared as described above. The lids of the two plates were then removed, and the fungal plate was inverted onto the *Bacillus* plate. The edges of the two plates were taped together with plastic paraffin film. Control

plates were similarly prepared except a non-inoculated plate was used instead of the *Bacillus* plate.

The three *in vitro* assays consisted of three replicates per treatment, with five plates per replicate. All plates were incubated at 22 °C for 5 d, and the inhibition of fungal growth was measured using the following formula:

Inhibition (%) =
$$\frac{C-T}{C} \times 100$$
,

where C = S. sclerotiorum growth on control plates and T = S. sclerotiorum growth on plates containing bacteria, CFCF, or volatile compounds. In all three assays, the number of sclerotia per plate was also recorded after an incubation for 15 d. The decrease in the number of sclerotia (as a percentage) was calculated relative to the control plates that were not inoculated with bacteria.

IV-2-5 Microscopy studies

For light microscopy studies, the inhibition zone on the dual-culture plates consisting of *S. sclerotiorum* and bacteria or bacterial compounds was examined using an inverted compound microscope. The same microscopic field was observed daily for up to 7 d. Similar samples were analyzed by scanning electron microscopy to evaluate the antagonistic effects of the bacterial isolates on *S. sclerotiorum* according to the method of Sharma and Sharma (2008). Approximately 2–3-mm mycelial segments with a thin layer of the upper part of the PDA were cut and rapidly placed in glass vials containing 3% glutaraldehyde, which had been prepared with 0.05 M phosphate buffer (pH 7.2). Samples were then fixed in this solution at 4 °C for 48 h, and washed with the same buffer. The samples were dehydrated in an ethanol series for 30 min at each dilution, and finally in absolute ethanol for 24 h. The dried samples were mounted over the stubs with double-stick adhesive carbon tape, and coated with gold using an Eiko IB-3 vacuum ion sputter coater (Eiko Engineering Co. Ltd., Tokyo, Japan) for about 3 min. Finally, samples were analyzed using a JSM-6510LA scanning electron microscope (JEOL Ltd., Tokyo, Japan) operating at 20 kV with different magnifications.

The effects of *Bacillus* isolates on *S. sclerotiorum* apothecia were also examined. Newly developed and small apothecia, which had been generated using the method described by Rahman et al. (2015), were used, including the stalks and sclerotia. The apothecia were soaked in a suspension containing actively growing bacteria for 24 h, and then placed on sterile moist soil in a Petri dish. After a 2-week incubation, apothecia were examined using an electron microscope. As controls, apothecia were soaked in sterilized water instead of a bacterial suspension. Ascospore germination tests were completed as described by Souto et al. (2004) using diluted (50%) cell-free supernatant. Samples were observed using a light microscope.

IV-2-6 Seed bacterization study

Surface-sterilized mustard seeds (Kibana-no-chikara, Takii Co., Kyoto, Japan) were immersed in diluted *Bacillus* cultures $[10^4 \text{ colony forming units (cfu) ml}^{-1}]$ for 30 min. According to a modified version of a method described by Abdullah et al. (2008), two 5-mm *S. sclerotiorum* mycelial plugs were placed on both sides of a PDA plate. Five bacterized seeds were then placed in a row between the two mycelial plugs. The experiments were completed with three replicates per treatment, with 10 plates per replicate. Untreated seeds were used as controls. After incubating samples at 25 °C for 3 and 7 d, the numbers of healthy germinated seeds and infected seedlings were recorded.

IV-2-7 Detached leaf inoculation assay

Detached mustard leaves of the same variety, age, size, and position were used. A single leaf was detached from each 3-week-old plant to ensure uniform leaves were used. Eight bacterial isolates were prepared from 48-h broth cultures by centrifugation at 3,000 rpm for 10 min. The pellets were resuspended in sterile distilled water amended with 0.05% Tween-20 and the concentrations were adjusted to $5\times10^8~\text{cfu}$ ml⁻¹. Detached leaves were soaked in a prepared bacterial suspension, and then air dried. Three treated leaves were placed on a glass slide and positioned on top of water-moistened paper in a 15-cm plastic Petri dish to avoid direct contact with water. Single 5-mm mycelial discs from PDA plates containing actively growing S. sclerotiorum were used to inoculate each leaf in the same position. Control leaves were soaked in sterilized distilled water before being inoculated with S. sclerotiorum. Plates were incubated in a growth chamber (Biotron) at 22 °C and 85% relative humidity, with a 12-h photoperiod under 12,000 lx (fluorescent lighting) for 1–5 d. The experiments were completed using three replicates, with nine leaves in each replicate. The area of infection on each detached leaf was determined based on the average infection area (%). The AUDPC was calculated using the following formula (Shaner and Finney, 1977):

$$AUDPC = \sum_{i=1}^{n} [(Y_{i+n1} + Y_i)/2] [X_{i+1} - X_i]$$

where Y_i = diseased area (%) at the ith observation, X_i = time (days) at the ith observation, and n = total number of observations. Finally, the decrease in the AUDPC value of the treated samples (relative to the AUDPC value of the controls) was calculated and recorded as a percentage.

IV-2-8 Suppression of white mold with a foliar spray of bacterial isolates

Mustard seedlings (Kibana-no-chikara, Takii Co.) were grown in a greenhouse to assess the efficacy of a foliar spray containing *Bacillus* isolates in suppressing white mold. Seeds were planted in pots filled with autoclaved commercially available potting soil. The mustard seedlings were watered regularly as needed. The upper meristems of V3-stage seedlings were removed to maintain the tri-foliar blades (Zhang and Xue, 2010). Bacillus isolates were prepared using the same procedure as that used in the detached leaf inoculation assays. The isolates were applied to V3stage mustard plants using a hand-pump sprayer at a rate of 5 ml per plant. After air drying for 1 h, the plants were inoculated with 5 ml homogenized S. sclerotiorum MAFF244850 mycelial suspension ($OD_{600} = 1.8$). Mustard plants inoculated with S. sclerotiorum alone were used as controls. The pots were randomly arranged in a growth chamber and incubated using a 12 h light (20,000 lx; fluorescent lighting)/22 °C and 12 h dark/18 °C cycle. The relative humidity was maintained at 95%. Fourteen days after inoculation, disease severities were evaluated using a 0-5 scale (Zhang and Xue, 2010), where 0 = no disease; 1 = few necrotic leaf lesions with a total diseased area < 5%; 2 = several leaf lesions with a total diseased area of 5–10%; 3 = many leaf lesions with a total diseased area of 11-30% (on leaves), and stem rot occurring on the upper stem parts; 4 = large leaf lesions with a total diseased area of 31-50%, and rotted upper stem parts; and 5 = large leaf lesions with a total diseased area of more than 50%, and stem rot progressing downward on dying plants. The experiment was performed twice, with three replicates, each consisting of 15 plants. Using the disease rating scale, the suppression of white mold was calculated according to the method of Idris et al. (2007) with the following formula:

Disease Suppression (%) =
$$\frac{A-B}{A} \times 100$$

where A = severity of white mold disease in the absence of bacterial isolates, and B = severity of white mold in the presence of bacterial isolates.

IV-2-9 Seed germination and seedling vigor

To assess seed germination rates, 25 ml bacterial inoculum (10^8 cfu ml⁻¹) and 0.1 g carboxymethyl cellulose were added to a 100-ml Erlenmeyer conical flask. Seeds (10 g) were surface sterilized and soaked in the bacterial suspension using a rotary shaker (150 rpm) for 30 min. The bacterial suspension was drained, and the seeds were aseptically dried overnight under laminar air flow. Ten seeds were placed on each solid agar plate. The experiment was completed in triplicate, and seeds soaked in sterile deionized water amended with carboxymethyl cellulose served as controls. All plates were incubated at 28 ± 2 °C for 7 d. The number of germinated seeds and length of radicles and plumules were recorded, and the seedling vigor index was calculated using the following formula (Abdul Baki and Anderson, 1973):

Seedling vigor index = Percent germination × seedling length (shoot length + root length)

IV-2-10 Statistical analysis

Data were analyzed using standard analysis of variance. The treatment means were separated at the 5% significance level using Tukey's multiple range tests.

IV-2-11 Nucleotide sequence accession numbers

Gene sequences were deposited in the DNA Data Bank of Japan with the accession numbers AB973460–AB973467, AB973469–AB973476, AB973478–AB973485,

AB973487–AB973494, and AB971633–AB971640 for *bacA*, *dfnM*, *fenA*, *ituA*, and *sfp*, respectively.

IV-3 Results

IV-3-1 Production of hydrolytic enzymes and plant growth-promoting compounds

In this study, we first investigated different modes of action, such as direct antibiosis through the production of antifungal enzymes, used by the eight isolates listed in Table 4. All *Bacillus* isolates exhibited protease activity, and all except RNB-107 were also positive for chitinase activity. All isolates except RNB-100 grew on minimal medium supplemented with laminarin azure, indicating they had β -1,3-glucanase activity. Cellulase activity was not detected.

We then investigated the plant growth-promoting attributes of the eight bacterial isolates. Isolates RNB-88, 92, 98, 113, 117, and 110 produced IAA (Table 4). Siderophore production, phosphate solubilization, and NH_3 production were also common among the isolates.

IV-3-2 Biofilm formation

All of the tested bacterial isolates formed biofilms, with variable OD_{630} values in a microtiter plate assay. Biofilm formation was highest for RNB-113, RNB-107, and RNB-88, followed by RNB-92. Biofilm formation decreased after an incubation for 72 h, except for RNB-98 and RNB-107 (Fig. 7).

IV-3-3 Polymerase chain reaction detection of antibiotic genes

Of the eight antibiotic genes screened, five (*bacA*, *dfnM*, *fenA*, *ituA*, and *sfp*) were detected in the eight *Bacillus* isolates (i.e., the corresponding bands were observed during agarose gel electrophoresis). The sequences of *bacA*, *dfnM*, and *sfp* were relatively conserved among the isolates (Fig. 8A, B, and E). In contrast, there was greater diversity in the *fenA* and *ituA* sequences (Fig. 8C and D).

IV-3-4 In vitro inhibition of Sclerotinia sclerotiorum

Decreased mycelial growth and inhibited sclerotia formation were observed in *in vitro* assays involving dual-cultures, CFCFs, and volatile compounds. *Bacillus* isolates produced distinct inhibition zones on PDA plates in dual-culture and CFCF assays (Fig. 9). Fungal growth was suppressed by 65.9–82.5% in dual-culture assays (Fig. 10A). Growth suppression was greatest (82.5%) with isolate RNB-88, followed by RNB-92 (82.0%), RNB-110 (75.3%), and RNB-117 (74.9%). The other four isolates, RNB-98 (71.3%), RNB-100 (65.9%), RNB-107 (66.8%), and RNB-113 (68.6%), had lower fungal growth suppression activities in the dual-culture assay.

The *Bacillus* CFCFs inhibited *S. sclerotiorum* growth by 38.3–62.2% (Fig. 10B). The CFCF from RNB-92 suppressed *S. sclerotiorum* growth the most (62.2%), followed by RNB-117 (59.4%), RNB-110 (57.8%), and RNB-88 (57.5%). Statistically similar growth suppression activities were observed for CFCFs from RNB-98 (50.8%), RNB-100 (46.1%), RNB-107 (40.6%), and RNB-113 (38.3%).

The eight *Bacillus* isolates produced a diverse range of volatile compounds that inhibited *S. sclerotiorum* mycelia (Fig. 10C). The highest levels of growth suppression were observed for the volatile compounds of RNB-92 (54.4%) and RNB-110 (53.3%). The volatile compounds from RNB-88 (51.0%), RNB-92 (54.4%), and RNB-110 (53.3%) exhibited similar growth suppression activities. The volatile

compounds from RNB-100 (34.0%) and RNB-107 (28.3%) were less inhibitory than those of the other bacterial isolates. Of the three *in vitro* assays that were completed, the overall trend regarding degree of fungal growth suppression was dual-culture assay > CFCF assay > volatile compound assay.

The production of sclerotia decreased in the presence of *Bacillus* isolates (Table 5) by 70.7–82.7%, 48.7–59.5%, and 48.9–66.0% in dual-culture, CFCF, and volatile compound assays, respectively. The decrease in the number of sclerotia was greatest in the dual-culture assay, followed by the volatile compound and CFCF assays.

IV-3-5 Microscopy studies

In the dual-culture experiments, no mycelial abnormalities were observed by microscopy after an incubation for 3 d (Fig. 11A-1). However, morphological abnormalities, such as lysis of mycelia, exudation of cytoplasmic materials, and blackening of mycelia, were detected after an incubation for 5 d (Fig. 11A-2). For all *Bacillus* spp., the degree of mycelial deformities increased from day 4 to 7. Scanning electron microscopy results confirmed the curling of mycelia, along with other mycelial abnormalities, including the breakdown of the cell wall (Fig. 11B and C). Control samples not exposed to bacterial isolates exhibited normal and actively growing hyphae. For all bacterial treatments, apothecia were abnormal or dead, and unable to produce ascospores (Fig. 12A). The control apothecia yielded ascospores as usual. Ascospore germination was normal following treatment with water (Fig. 12B), while cell-free supernatants from all *Bacillus* isolates inhibited ascospore germination (Fig. 12C).

IV-3-6 Seed bacterization study

Seed bacterization with *Bacillus* isolates offered protection against *S. sclerotiorum* infection (Fig. 13, Table 6). The protection was highest for RNB-92 (98.3%), followed by RNB-110 (97.7%) and RNB-88 (95.3%). The lowest level of protection was provided by RNB-107 (87.3%). Seed rot was avoided even with abundant *S. sclerotiorum* inoculum from both sides of a PDA plate. In contrast, control seedlings that were not treated with bacterial isolates were infected by the fungus after an incubation for 7 d (Fig. 14A and B).

IV-3-7 Detached leaf inoculation assay

In the detached leaf assay, disease suppression by the *Bacillus* isolates was evaluated based on the AUDPC value (Fig. 13B and Table 6). The AUDPC values of treated samples decreased to 71.6–91.1% of those of the controls. The AUDPC value decreased the most (91.1%) with RNB-110, followed by RNB-92 (90.7%), RNB-88 (90.0%), and RNB-113 (87.4%). The onset of *S. sclerotiorum* infection occurred later on detached leaves treated with bacterial cell suspensions than on untreated control leaves (Fig. 15A and B).

IV-3-8 Suppression of white mold with a foliar spray of bacterial isolates

The suppression of white mold of mustard using a foliar spray containing *Bacillus* isolates (RNB-88, RNB-92, RNB-110, and RNB-113) ranged from 80.0–90.0% (Fig. 13C and Table 7). White mold symptoms decreased the most (90.0%) following the application of RNB-92. Treatment with RNB-88, RNB-110, and RNB-113 decreased white mold symptoms by 88.5, 87.1, and 80.1%, respectively. Leaves and plants treated with the *Bacillus* isolates remained healthy after being inoculated with *S. sclerotiorum*. Conversely, the leaves and stems of plants infected by the fungus

without a prior bacterial treatment rotted, resulting in the highest disease rating score (i.e., 5 on a 0–5 scale) (Fig. 15C and D).

IV-3-9 Seed germination and seedling vigor

Plant growth was promoted and an increase in the seed germination rate was observed following seed bacterization (Table 8). The seed germination rates (%) were statistically similar for all treatments, and were 25.3–30.2% higher than the germination rate for the control seeds. Regarding seedling vigor, similar index values were observed following treatment with RNB-113 (700), RNB-92 (675), RNB-98 (672), RNB-88 (668), and RNB-117 (665). These values were higher than that observed for RNB-110 (624). The seedling vigor index increased by 30.8–87.2% following treatment with *Bacillus* isolates, suggesting the bacteria had growth-promoting attributes (Table 8).

Table 3. Primers used to identify genes involved in the biosynthesis of antifungal

compounds

		1 (C)
acAF1	GTGAAGGCCGTACTTTTGTCTGGC	55
acAR1	GGGGGGAAATACAGCTTCAGGGC	
eaBF1	GCCCGAAACGGCAGCGCCTG	60
eaBR1	CGGAATGGAGGCTTTGATCCTCTG	
fnMF1	CGGAGTGAAACCGTGCCGGGATAAAGA	55
fnMR1	GACCATTCAGAGCGGAAAGCTCC	
hbFF3	GCCTAGATGACATGGCGGCGG	55
hbFR2	GCCGCCGTAGTCGTCCGTGAAGACCG	
enAF1	CCTCGCTCCGCATGATCTTTTGG	60
enAR1	CGGGAGCACGGTGGCAATGTG	
u-P1-F	AGCTTAGGGAACAATTGTCATCGGGGGCTTC	60
u-P2-R	TCAGATAGGCCGCCATATCGGAATGATTCG	
fp-P17-F	ATGAAGATTTACGGAATTTA	46
fp-P18-R	TTATAAAAGCTCTTCGTACG	
rf-F	CTAGAATTCAGATTTACGGAATTTATATG	56
rf-R	GGGGAATTCAGGGTGTGCGGCGCATAC	
	acAF1 acAR1 aaBF1 aaBR1 abFF3 abFF3 abFF3 abFR2 anAF1 anAF1 anAF1 a-P1-F a-P2-R p-P17-F p-P18-R f-F f-R	acAF1GTGAAGGCCGTACTTTTGTCTGGCacAR1GGGGGGAAATACAGCTTCAGGGCacAR1GGCCGAAACGGCAGCGCCTGcaBF1GCCCGAAACGGCAGCGCCTGcaBR1CGGAATGGAGGCTTTGATCCTCTGinMF1CGGAGTGAAACCGTGCCGGGATAAAGAinMR1GACCATTCAGAGCGGAAAGCTCCabFF3GCCTAGATGACATGGCGGCGGabFF2GCCGCCGTAGTCGTCCGTGAAGACCGnAF1CCTCGCTCCGCATGATCTTTTGGnAR1CGGGAGCACGGTGGCAATGTGa-P1-FAGCTTAGGGAACAATTGTCATCGGGGCTTCa-P2-RTCAGATAGGCCGCCATATCGGAATGATTCGp-P17-FATGAAGATTTACGGAATTTAp-P18-RTTATAAAAGCTCTTCGTACGf-FCTAGAATTCAGATTTACGGAATTTATATGf-RGGGGAATTCAGGGTGTGCGGCGCATAC

[¶]Annealing temperature for polymerase chain reaction

	Antifungal enzyme production				Plant growth-promotional attributes			
Isolates	Protease	Chitinase	β-1,3-	Cellulase	IAA	Siderophore	Р	NH ₃
			Glucanase		production	formation	solubilization	production
RNB- 88	+	+	+	-	+	+	+	+
RNB-92	+	+	+	-	+	+	+	+
RNB-98	+	+	+	-	+	+	+	+
RNB-100	+	+	-	-	-	+	+	+
RNB-107	+	-	+	-	-	+	+	+
RNB-110	+	+	+	-	+	+	+	+
RNB-113	+	+	+	-	+	+	+	+
RNB-117	+	+	+	-	+	+	+	+

Table 4. Antifungal enzyme production and plant growth-promoting attributes of eight *Bacillus* isolates

+, presence; -, absence; IAA, indoleacetic acid; P solubilization, phosphate solubilization

	Suppression of sclerotia formation (%)			
Isolates	Dual	CFCF	Volatile	
RNB-88	82.7 ± 0.9 a	55.4 ± 7.4 ab	66.0 ± 1.3 a	
RNB-92	80.8 ± 3.0 a	$54.9 \pm 3.8 \text{ ab}$	$60.4 \pm 0.9 \text{ ab}$	
RNB-98	$70.7\pm6.0\ b$	$53.6 \pm 2.8 \text{ ab}$	63.9 ± 7.5 a	
RNB-100	77.5 ± 4.4 a	52.5 ± 3.1 ab	$64.3 \pm 6.8 \text{ a}$	
RNB-107	81.4 ± 2.8 a	$48.7\pm10.6~\text{b}$	$60.1 \pm 2.6 \text{ ab}$	
RNB-110	80.1 ± 2.4 a	53.3 ± 2.5 ab	$64.1 \pm 6.2 \text{ a}$	
RNB-113	81.3 ± 3.2 a	59.5 ± 5.7 a	61.8 ± 9.7 a	
RNB-117	$76.8 \pm 3.4 \text{ ab}$	$55.9\pm0.4\ ab$	$48.9\pm5.9~b$	

Table 5. Suppression of sclerotia production by Sclerotinia sclerotiorumMAFF244850 following treatment with Bacillus isolates in three assays

Dual, dual-culture assay; CFCF, cell-free culture filtrate assay; Volatile, volatile compound assay. The results are presented as the mean \pm standard deviation from three replicates.

Mean values followed by the same letter within columns are not significantly different (P < 0.05) according to Tukey's test.

Isolates	Seed bacterization	Detached leaf assay
RNB-88	95.33 ± 5.03 ab	89.96 ± 1.38 a
RNB-92	98.33 ± 2.89 a	90.71 ± 1.82 a
RNB-98	90.67 ± 5.13 ab	$74.77\pm8.80\ b$
RNB-100	92.33 ± 7.51 ab	80.24 ± 10.07 ab
RNB-107	$87.33 \pm 2.08 \text{ b}$	$80.88 \pm 5.93 \text{ ab}$
RNB-110	97.67 ± 2.52 a	91.10 ± 2.75 a
RNB-113	93.67 ± 5.51 ab	87.42 ± 3.28 a
RNB-117	92.00 ± 3.61 ab	71.55 ± 5.64 b

Table 6. Suppression (%) of seedling rot, and leaf rot using Bacillus isolates during

seed bacterization, and detached leaf assay

The results are presented as the mean \pm standard deviation from three replicates. Mean values followed by the same letter within columns are not significantly different (P < 0.05) according to Tukey's test.

 Table 7. Suppression (%) of white mold of mustard using lead *Bacillus* isolates

 during foliar spray

Isolates	Disease suppression
RNB-88	88.51 ± 2.79 a
RNB-92	90.03 ± 2.22 a
RNB-110	87.12 ± 4.22 a
RNB-113	$80.05 \pm 1.53 \text{ b}$

The results are presented as the mean \pm standard deviation from three replicates. Mean values followed by the same letter within columns are not significantly different (P < 0.05) according to Tukey's test.

 Table 8. Mustard seed germination and seedling vigor following seed bacterization

 with Bacillus isolates

Strains	Germination %	Seedling vigor
RNB-88	98.0 a	668.2 ab
RNB-92	98.3 a	675.3 ab
RNB-98	97.7 a	672.1 ab
RNB-100	96.7 a	499.5 c
RNB-107	94.3 a	488.9 c
RNB-110	96.0 a	623.5 b
RNB-113	98.3 a	700.2 a
RNB-117	96.0 a	664.7 ab
Control	76.7 b	328.7 d



Fig. 7. Biofilm formation in Luria-Bertani medium during a microtiter plate assay with eight *Bacillus* isolates.



Fig. 8. Phylogenetic trees for five antibiotic genes in *Bacillus* isolates. Numbers in parentheses indicate the accession numbers in the GenBank database. Bar: sequence divergence



Fig. 9. Suppression of *Sclerotinia sclerotiorum* MAFF244850 mycelial growth and sclerotia production by *Bacillus* isolate RNB-92 in dual-culture and cell-free culture filtrate (CFCF) assays. (A) The inhibition of mycelial growth was observed as a distinct inhibition zone surrounding the bacteria on a dual-culture plate. (B) There was no inhibition zone on the dual-culture assay control plate. (C) Mycelial growth was inhibited surrounding the holes containing CFCF, while (D) the mycelia covered the entire control plate.



Fig. 10. Suppression of *Sclerotinia sclerotiorum* growth (%) by *Bacillus* isolates in (A) dual-culture, (B) CFCF, and (C) volatile compound assays.



Fig. 11. Microscopy results for the antagonistic effects of *Bacillus* isolate RNB-92 against *S. sclerotiorum* MAFF244850 in a dual-culture assay. (A-1) Normal mycelia after an incubation for 3 d, and (A-2) mycelial tip and cell wall lysis with blackening of hyphae after an incubation for 5 d, in the same examination field observed using an inverted compound microscope. (B) Mycelial tip lysis with curling and (C) mycelial cell wall breakdown were also observed with a scanning electron microscope (JEOL, JSM-6510LA) after an incubation for 5 d.



Fig. 12. Antagonistic effects of *Bacillus* isolates on an ascocarp and ascospores. (A) Abnormal or dead apothecia after treatment with a diluted bacterial culture. (B) Germination of an untreated ascospore and (C) inhibition of ascospore germination following treatment with a cell-free supernatant.



Fig. 13. Suppression (%) of seedling rot, leaf rot, and white mold using *Bacillus* isolates during (A) seed bacterization, and in (B) a detached leaf assay and (C) foliar spray.





Fig. 14. Control of seedling rot using *Bacillus* **isolates.** (**A**) Seed bacterization with *Bacillus* RNB-92 inhibited *S. sclerotiorum* mycelial growth and prevented seedling rot. (**B**) Seedling rot developed and sclerotia were produced on the control plate.



Fig. 15. Biocontrol of white mold of mustard using *Bacillus* isolates. (A) Detached mustard leaves treated with *Bacillus* isolates exhibited no disease symptoms 72 h after inoculation with *Sclerotinia sclerotiorum*. (B) Rotting from the inoculation point was observed on control leaves. (C) Disease symptoms were not observed in *S. sclerotiorum*-infected mustard plants treated with *Bacillus* RNB-92. (D) Leaf and stem rot as well as leaf-drop symptoms were observed in *S. sclerotiorum*-infected control plants.

Chapter V

Discussion

V-1 Characterization of bacterial isolates

This study involved eight bacterial isolates with inhibitory activities against *S. sclerotiorum*. Phenotypic characteristics and 16S rDNA sequences confirmed that all of the isolates belonged to the *Bacillus* genus (Fig. 5). Although 16S rDNA sequencing is a powerful tool for identifying bacteria and determining phylogenetic and evolutionary relationships (Weisburg et al., 1991), it can be unreliable when examining closely related taxa. Protein-coding genes exhibiting high genetic variation can be used for more accurate classifications and identifications. Therefore, the *gyrA* gene of the *Bacillus* isolates was partially sequenced (Chun and Bae, 2000), which confirmed that all isolates were *B. amyloliquefaciens* subsp. *plantarum* (Fig. 6).

The eight *B. amyloliquefaciens* isolates produced endospores, and were resistant to heat (80 °C) and salinity stresses, consistent with previous findings that indicated *Bacillus* isolates can survive in adverse environmental conditions (Pérez-García et al., 2011). Various diseases caused by fungi, bacteria, systematic viruses, and nematodes on a range of plant hosts have been controlled with the application of several *Bacillus* spp., including *B. amyloliquefaciens*, *B. subtilis*, *B. cereus*, and *B. pumilis* (Choudhary and Johri, 2009; Kloepper et al., 2004). The biocontrol activities exhibited by *Bacillus* spp. are mainly through direct antagonism of phytopathogens (Yu et al., 2002) and stimulation of plant host defenses through the induction of systemic resistance (Kloepper et al., 2004; Whipps, 2001; Choudhary and Johri 2009).

V-2 Biological control mode of action

The production of lytic enzymes to hydrolyze the cell wall of spores and fungal hyphae was analyzed (Table 3 and Fig. 11). Chitinase was produced by seven of the eight isolates. This enzyme hydrolyzes chitin to oligomeric and monomeric

components through the breakdown of glycosides. Chitinases are important constituents of several bacterial species, including *Bacillus* spp., in the biological control of fungal pathogens (e.g., Singh et al., 1999). Similar to chitinase, β -1,3-glucanase is a potent enzyme used to control fungal pathogens. It is also produced by several microorganisms, including *Bacillus* spp. (Leelasuphakul et al., 2006). Purified forms of such metabolites and lytic enzymes can inhibit the mycelial growth of certain fungi (Leelasuphakul et al., 2006). In this study, we did not observe cellulase production (Table 3), which is consistent with the findings of Cazorla et al. (2007). Extracellular lytic enzymes, such as proteases, protect fungi, including *Pythium ultimum*, the causal agent of damping disease of sugarbeet (Dunne et al., 1997).

Indoleacetic acid was produced by the *Bacillus* isolates in an *in vitro* test that used tryptophan as a precursor for IAA biosynthesis (Table 3). Similar results were obtained for *Bacillus* isolates by Ahmad et al. (2008). According to the findings of Minaxi et al. (2012), the production of IAA is much greater in the presence of tryptophan. Ammonia production has also been reported as a common trait of several plant growth-promoting rhizobacteria (Ahmad et al., 2008).

The eight *Bacillus* isolates used in this study were able to solubilize phosphate and generate siderophores (Table 3). Ahmad et al. (2008) reported that 80% of tested *Bacillus* isolates have phosphate-solubilizing activities. Siderophores produced by rhizospheric bacteria increase the availability of iron near roots, which can enhance plant growth (Alexander and Zuberer, 1991). Additionally, rhizobacteria can protect plants from disease by producing a range of siderophores to make iron unavailable, which inhibits the growth of pathogenic fungi (Whips, 2001).
All of the *Bacillus* isolates formed biofilms (Fig. 7). A surfactin-producing biofilm is expected to inhibit other microbes, and has been suggested as an important factor for the colonization of root and leaf surfaces by *Bacillus* isolates (Pérez-García et al., 2011). Stable biofilms and the secretion of surfactin act together to protect plants from pathogenic microbes (Stein, 2005; Ongena and Jacques, 2007).

The PCR results confirmed that the isolates possessed multiple genes for metabolites involved in disease suppression by Bacillus spp. (Fig. 8). Approximately 4-5% of the B. subtilis genome consists of antibiotic genes (Stien, 2005), while about 8% of the B. amyloliquefaciens genome is associated with antibiotic biosynthesis (Arguelles-Arias et al., 2009; Chen et al., 2009a). Various biologically active molecules synthesized by Bacillus spp. have been reported to suppress the growth and development of different fungal phytopathogens through hyphal cell perturbation and/or permeabilization of spores or conidia, resulting in germination failure (Zhao et al., 2014). Three families of lipopeptides (i.e., surfactins, iturins, and fengycins) have been studied mainly regarding their antagonistic activities toward a wide range of phytopathogens (Ongena and Jacques, 2007). Additionally, antibacterial polyketides, such as difficidin, and the dipeptide antibiotic bacilysin, are involved in the biocontrol of plant pathogens (Arguelles-Arias et al., 2009). Surfactin, which is a potent biosurfactant, has hemolytic and antimicrobial activities, and can form biofilms (Ongena and Jacques, 2007). Antibiotic lipopeptides, such as iturin, can inhibit the growth of several pathogenic fungi (e.g., Yu et al., 2002). Fengycin from *Bacillus* spp. can efficiently suppress the growth and development of filamentous fungi, including Sclerotinia spp. (Hou et al., 2006) and Fusarium spp. (Zhao et al., 2014). Difficidin has broadspectrum antibacterial activity. Bacilysin and difficidin can inhibit the growth of Erwinia amylovora (Chen et al., 2009b). Moreover, potential cyclic lipopeptides (e.g.,

surfactins and fengycins) are elicitors of induced systemic resistance (Ongena and Jacques, 2007).

V-3 In vitro biocontrol of Sclerotinia sclerotiorum

All eight screened *Bacillus* isolates had high inhibitory activities against *S. sclerotiorum* in the dual-culture assay (Fig. 10A). There were clear inhibition zones (> 0.5 cm), which were similar to those observed during the initial screening to identify effective isolates (Table 1). The *Bacillus* isolates could inhibit mycelial growth *in vitro* by up to 82.5% in the dual-culture assay. These results concur with those of another study that involved a dual-culture assay (Souto et al., 2004). Therefore, these types of assays may be important tools for evaluating fungal growth suppression with bacterial biocontrol agents.

The formation of an inhibition zone (Fig. 9) was due to the excretion of antimicrobial metabolites (Kumar et al., 2012). Changes in mycelial color during suppressed growth were observed during the co-culture of *S. sclerotiorum* with antagonistic bacteria. Similar observations regarding the retardation of *in vitro S. sclerotiorum* growth and mycelial discoloration with suppressed germ tube formation were reported by Yang et al. (2009) and Sharma and Sharma (2008).

An inhibition zone was observed during the dual-culture and CFCF assays (Fig. 10A and B), which indicated the presence of biologically active metabolites that diffused through the agar medium. According to Yoshida et al. (2001), *B. amyloliquefaciens* culture filtrate can inhibit the *in vitro* growth of several phytopathogenic fungi and bacteria such as *Rosellinia necatrix*, *Pyricularia oryzae*, *S. sclerotiorum*, *Agrobacterium tumefaciens*, and *Xanthomonas campestris* pv. *campestris*. Thus, culture filtrates may offer protection against disease development.

Antagonistic bacterial volatile compounds adversely affected fungal growth and development (Fig. 10C). Interactions with *B. subtilis* volatile compounds produced morphologically abnormal hyphae and conidia in some plant pathogens (Chaurasia et al., 2005). In the present study, the overall growth suppression during the dual-culture and CFCF assays was higher than that observed in the volatile compound assay. These results differed from those of Chaurasia et al. (2005).

V-4 Microscopy results regarding biocontrol of *Sclerotinia sclerotiorum* by *Bacillus* isolates

Microscopy results for the interaction between *Bacillus* isolates and *S. sclerotiorum* revealed morphological abnormalities in fungal structures (Fig. 11 and 12). Lysis of hyphae and cytoplasmic exudations were observed when *S. sclerotiorum* was challenged with the eight *Bacillus* isolates. These observations are supported by the results of studies conducted by Abdullah et al. (2008) and Chaurasia et al. (2005). Morphological changes, including twisting and coiling of mycelia and pore formation in the mycelial wall of *S. sclerotiorum*, have occurred during interactions with *B. subtilis* (Sharma and Sharma, 2008; Yang et al., 2009). Although there is no report regarding the direct inhibition of apothecia, the failure of ascospores to germinate after exposure to antifungal metabolites from *Bacillus* spp. has been described (Souto et al. 2004).

V-5 Seed bacterization and plant applications

Our observations regarding seed bacterization (Figs. 13A and 14) are consistent with those of another study, which reported improved germination and suppressed seedling diseases in various crops following treatments with *Bacillus* isolates (Zheng and

Sinclair, 2000). Seed treatment with *Bacillus* isolates reduced disease incidence by up to 65% when co-inoculated with *Colletotrichum gloeosporioides* (Ashwini and Srividay, 2013).

Bacillus isolates substantially decreased *S. sclerotiorum* growth during the detached leaf assay (Figs. 13B and 15A). In another report, *B. subtilis* reduced *Alternaria* spp. spore germination on mustard leaves in an *in vitro* test (Sharma and Sharma, 2008).

Four bacterial isolates were identified as being able to suppress white mold of mustard in growth chamber experiments (Figs. 13C and 15C). The observed disease suppression might involve the inhibition of inocula and enhancement of induced systemic resistance. Hu et al. (2005) observed a significant reduction in the incidence of *S. sclerotiorum* diseases in oilseed rape plants treated with *B. subtilis* Tu-100 under artificial infection conditions. Furthermore, the foliar application of *B. subtilis* on soybean plants under controlled conditions effectively reduced stem rot caused by *S. sclerotiorum* (Zhang and Xue, 2010).

V-6 Plant growth promotion

The ability of bacilli to promote plant growth is well documented. This study also revealed that mustard seed germination and seedling vigor improved following seed bacterization with the *Bacillus* isolates (Table 5). *Bacillus* populations increase the bioavailability of mineral nutrients and essential compounds in the rhizosphere. Several *Bacillus* spp., including *B. amyloliquefaciens*, promote IAA production, which may stimulate root growth and nutrient uptake (Pérez-García et al., 2011). Beneduzi et al. (2012) reported that bacteria of diverse genera were effective promoters of plant growth, with *Bacillus* spp. forming one of the main groups. Plant growth promotion and the elicitation of induced systemic resistance against various

phytopathogens have been reported for plant-associated bacilli, such as *B. amyloliquefaciens*, *B. subtilis*, *B. cereus*, *B. pumilus*, and *B. mycoides* (Choudhary and Johri, 2009; Kloepper et al., 2004).

V-7 Conclusion

The isolated *Bacillus* spp. effectively suppressed the growth of *S. sclerotiorum* and the formation of sclerotia in *in vitro* and *in vivo* assays. The efficacy of biological control agents depends on their reliability in field conditions because of the high variability in efficacy, survival, and production of metabolites.

V-8 Future research

The *Bacillus* isolates should be tested in field trials under different environmental conditions. As living organisms, bacteria need suitable environmental conditions for their growth and development. The development of suitable formulations is also important for the commercialization of any biocontrol agent. Therefore, assessing the use of appropriate carrier materials and viability of the *Bacillus* spp. in formulations is necessary before any biocontrol agent can be commercialized.

Chapter VI

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