Transcriptional Profiling of Diffusible Lipopeptides and Fungal Virulence Genes During *Bacillus amyloliquefaciens* EZ1509-Mediated Suppression of *Sclerotinia sclerotiorum*

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Accepted for publication 17 July 2019.

ABSTRACT

Sclerotinia sclerotiorum is a devastating necrotrophic pathogen that infects multiple crops, and its control is an unremitting challenge. In this work, we attempted to gain insights into the pivotal role of lipopeptides (LPs) in the antifungal activity of *Bacillus amyloliquefaciens* EZ1509. In a comparative study involving five *Bacillus* strains, *B. amyloliquefaciens* EZ1509 harboring four LPs biosynthetic genes (viz. surfactin, iturin, fengycin, and bacilysin) exhibited promising antifungal activity against *S. sclerotiorum* in a dual-culture assay. Our data demonstrated a remarkable upsurge in LPs biosynthetic gene expression through quantitative reverse transcription PCR during in vitro interaction assay with *S. sclerotiorum*. Maximum upregulation in LPs biosynthetic genes was observed on the second and third days of in vitro interaction, with iturin and fengycin being the highly expressed genes. Subsequently, Matrix-assisted laser desorption/ionization-time of flight-

mass spectrometry analysis confirmed the presence of LPs in the inhibition zone. Scanning electron microscope analysis showed disintegration, shrinkage, plasmolysis, and breakdown of fungal hyphae. During in planta evaluation, *S. sclerotiorum* previously challenged with EZ1509 showed significant suppression in pathogenicity on detached leaves of tobacco and rapeseed. The oxalic acid synthesis was also significantly reduced in *S. sclerotiorum* previously confronted with antagonistic bacterium. The expression of major virulence genes of *S. sclerotiorum*, including endopolygalacturonase-3, oxalic acid hydrolase, and endopolygalacturonase-6, was significantly downregulated during in vitro confrontation with EZ1509.

Keywords: disease control and pest management

Sclerotinia sclerotiorum is a soilborne fungus that causes serious losses to economically important crops worldwide (Boland and Hall 1994). The ability of this fungus to produce sclerotia that serve as survival structures for several years under harsh environment and infection propagules in the soil plays a key role in the pathogenicity (Bloomfield and Alexander 1967). However, oxalic acid produced by this pathogen is well documented to be the primary determinant of its virulence (Bolton et al. 2006; Kim et al. 2008). After successful colonization of the host plant, the pathogen produces cell wall-degrading enzymes (CWDEs) that disintegrate the cell wall and contribute to its virulence (Purdy 1979). *S. sclerotiorum* produces oxalic acid during early stages of host plant infection that creates an acidic environment and triggers the synthesis of CWDEs that are functionally active at low pH (Liang et al. 2015; Williams et al. 2011). Oxalic acid induces cell death in host plant supporting the necrotrophic nature of this pathogen. Oxalic acid-deficient mutants of *S. sclerotiorum* showed reduced virulence on host plants, supporting its vital role in pathogenicity (Liang et al. 2015). Therefore, the diverse array of infection structures and compounds produced by this pathogen signifies its importance as a potential threat to crop production.

Several control strategies, such as crop rotation, resistant germplasm, and moisture maintenance, have failed to control this pathogen because of lack of resistant varieties and the survival potential of sclerotia for an indefinite period in the soil. The most frequent control of *S. sclerotiorum* is through synthetic fungicides. However, the use of chemicals contributes to environmental hazards and health safety concerns (Boland and Hall 1987). Biological control has emerged as a sustainable and effective approach to control plant pathogens (Farzand et al. 2019; Massawe et al. 2018; Minuto et al. 2006). Among biological control agents, *Bacillus* species are promising bacteria that have been used to control several plant diseases (Hobley et al. 2013; Ongena and Jacques 2008; Stein 2005). The mechanisms through which *Bacillus* species affect the pathogen development generally include the following: (i) direct antagonism of the pathogen, (ii) competition for food and ecological niche, and (iii) release of toxic antibiotic substances (Whipps 2001). However, the primary mode of action of *Bacillus* species to suppress plant pathogenic fungi is antibiosis (Romero et al. 2007).

Bacillus species, particularly *Bacillus amyloliquefaciens* strains, are considered as ecofriendly, with excellent colonization capacity and versatile mode of action to suppress plant pathogens (Zhao et al. 2014). *B. amyloliquefaciens* dedicates up to 340 kb, which correspond to 8% of its genomic makeup, to produce a wide range of antimicrobial compounds, such as antibiotics, lytic enzymes, and several nonribosomally synthesized cyclic lipopeptides (LPs) and polyketides (Chen et al. 2009). Among these antimicrobial compounds, LPs have distinguished antimicrobial activity. They have been classified in several families, and among them, surfactin, fengycin, and iturin families are of high interest, because they can be secreted in high amounts under natural growth conditions of the rhizosphere (Debois et al. 2014; Nihorimbere et al. 2012). Surfactin

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Funding: This research was supported by National Key Research and Development Program of China grant 2017YFD0200400, National Natural Science Foundation of China grant 31471811, and Jiangsu Independent Innovation Fund for Agricultural Science and Technology grant CX(18)1003.

The author(s) declare no conflict of interest.

shows antimicrobial, antifungal, hemolytic, and antiviral activity by altering biological membrane integrity (Vater et al. 2003; Wang et al. 2011). Surfactin in combination with iturin has showed strong antifungal activity (Maget-Dana et al. 1992). Fengycin exhibited antifungal activity more specifically against filamentous fungi (Liu et al. 2011; Vanittanakom et al. 1986). Bacilysin is also an important LP with antibacterial and antifungal activity (Chen et al. 2009; Wu et al. 2015a, b). These compounds are thus crucial for rhizosphere fitness and biological control potential of *Bacillus* strains (Cawoy et al. 2015; Raaijmakers et al. 2010; Romero et al. 2007).

Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) is considered to be a more rapid and accurate tool to study the synthesis of LPs compared with conventional methods. The efficacy of MALDI-TOF-MS to identify cyclic LPs has been well documented (Kim et al. 2010; Vater et al. 2003). In this context, this study was conducted with a goal to emphasize the importance of antibiosis in direct antagonism of *S. sclerotiorum* and identify antifungal LPs involved in the direct antagonistic activity of *B. amyloliquefaciens* EZ1509 against the fungal pathogen. This work reveals that the LP-producing *B. amyloliquefaciens* EZ1509 had direct antifungal activity during *in vitro* confrontation, causing ultrastructural changes in fungal hyphae, reduction in oxalic acid production, and pathogenicity of *S. sclerotiorum* by downregulating the expression of virulence genes.

MATERIALS AND METHODS

Microbial cultures and growth conditions. *S. sclerotiorum* and *Bacillus* strains used in this study were procured from the Laboratory of Biological Control and Bacterial Molecular Biology, Nanjing Agricultural University, Jiangsu, China (Table 1). Bacterial strains were maintained on Luria Bertani (LB) broth with 60% (vol/vol) glycerol solution at -80°C . These cultures of bacterial strains were refreshed in solid LB medium, and then, a single colony was inoculated on liquid LB medium and incubated at 37°C . *S. sclerotiorum* culture was preserved in 60% glycerol and stored at -80°C ; this culture was refreshed on potato dextrose agar (PDA) plates and incubated for 4 days at 25°C for further use.

Screening for antagonistic activity. The inhibitory effect of five *Bacillus* strains on mycelial growth of *S. sclerotiorum* was tested in dual culture. A mycelial plug (0.6 cm) from a 4-day-old culture of *S. sclerotiorum* was placed at the center of the plate containing PDA medium (HiMEDIA, M096). Afterward, 5 μl of bacterial suspension from each *Bacillus* strain having optical density (OD) 2.50 measured at 600 nm was poured on sterilized

filter paper 3 cm away from the mycelial plug. The plates were incubated at 25°C for 4 days, and the diameter of the inhibition zone was recorded. The experiment was repeated three times, with three replicates of each treatment.

PCR amplification of LPs biosynthetic genes from genomic DNA. Genomic DNA from *B. amyloliquefaciens* EZ1509 was isolated by using a bacterial genomic DNA extraction kit (OMEGA Bio-tek) according to the manufacturer's manual. The concentration and purity of DNA were measured on NanoDrop 1000 (Thermo Scientific). DNA was amplified based on specifically designed primers for antifungal LP genes (viz. surfactin, iturin, fengycin, and bacilysin) (Table 2). The specificity of the primers was checked through NCBI nucleotide BLAST tools (<https://www.ncbi.nlm.nih.gov/>). PCR amplification of genomic DNA was carried out by using 2 $\times$ Rapid taq Mater mix (Vazyme) as per the manufacturer's protocol. The PCR conditions were as follows: initial denaturation at 95°C for 5 min, including 32 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, extension at 72°C for 30 s/kb, and a final extension at 72°C for 5 min. A negative control (without DNA) was also included in a PCR reaction. Amplified PCR product from each reaction was visualized on 1% agarose gel stained with ethidium bromide.

In vitro interaction assay. Antagonism of *B. amyloliquefaciens* EZ1509 against *S. sclerotiorum* was assessed by the previously described method of Johnson and Curl (1972). A 0.6-cm mycelial plug from the margins of an actively growing 4-day-old culture of *S. sclerotiorum* was placed at the center of the plate containing PDA medium. *B. amyloliquefaciens* EZ1509 ($\text{OD}_{600} = 2.5$) was streaked at a 3-cm distance from the *S. sclerotiorum* mycelial plug on both sides of the fungal plug and incubated in dark at 25°C for 4 days. In the control treatment, a mycelial plug of *S. sclerotiorum* was placed at the center of the plate containing PDA medium without the genus *Bacillus*. Each treatment was replicated three times, and the experiment was repeated three times.

Extraction of LPs from solid PDA medium. The production of LPs by *B. amyloliquefaciens* EZ1509 on solid PDA media during coculturing with *S. sclerotiorum* was examined following a predescribed protocol of Torres et al. (2016). Briefly, 1-cm blocks of PDA medium were taken from the inhibition zone close to the bacterial colony (ZCBC) and from the inhibition zone close to the fungal colony (ZCFC), and they were resuspended in 1 ml of acetonitrile. Samples for control were taken from plates containing bacterial antagonist without fungal pathogen. Samples were shaken vigorously for 1 min and centrifuged at 7,000 rpm for 5 min. The samples were dried in a speed vacuum evaporator (Labogene Aps indusfrivj 6.8 DK-3540 Lyng), redissolved in 50 μl of acetonitrile, and saved for MALDI-TOF analysis. The experiment was repeated three times, with three replicates of each treatment.

MALDI-TOF-MS. Mass spectra of LP extracts from solid PDA medium during coculturing were obtained through the Bruker-Daltonic instrument (MALDI-TOF-MS) at positive ion mode. The samples were first dried on a stainless steel target plate under a lamp for recording mass spectra using 2,5-dihydroxybenzoic acid as matrix solution. The voltage of reflector was set at 20 kV, and laser excitation was carried out at 337 nm with 80 shots.

TABLE 1. List of *Bacillus* strains used in this study

Strain identification	Name
EZ1509	<i>Bacillus amyloliquefaciens</i>
FZB42	<i>Bacillus amyloliquefaciens</i>
168	<i>Bacillus subtilis</i>
NMTD17	<i>Bacillus</i> sp.
LLCG23	<i>Bacillus wiedmannii</i>

TABLE 2. Primers used to amplify lipopeptides biosynthetic genes through PCR

Lipopeptides	Melting temperature	Size (base pairs)	Primers
Surfactin	56.3	1,298	CGGTGATCTTGCGAAGCTTTAT CGCTTTTCGTTCTGCCATTCT
Fengycin	56.3	1,447	CGGCCATTGCGTCATCTTTAT GTTTCCGCTTCATCAGTCTCTTC
Iturin	55.9	1,244	ACCTCACCTTGATCGGCTATAC TGGTGGGCGAAAGAAGTTTATG
Bacilysin	52.8	657	TCATGACTCTTCGCCTCT GAATGGGATAACGGAGTAAGAC

Ultrastructural changes in *S. sclerotiorum* mycelium.

Ultrastructural changes in *S. sclerotiorum* mycelium because of biocontrol activity of *B. amyloliquefaciens* EZ1509 were studied through scanning electron microscope (SEM) analysis (Sharma and Sharma 2008). An ~0.6-cm block of fungal mycelium with a thin PDA medium layer was excised from the edge of the inhibition zone, and the sample for control was taken from the edge of mycelial growth without bacterial antagonist. Both samples were placed in 2.5% (vol/vol) glutaraldehyde solution prepared in 0.05 M phosphate buffer (pH 7.2) and incubated for 24 h at 4°C after fixation. Later, mycelial growth was washed with the same buffer and dehydrated with a serial dilution of ethanol (30 min for each dilution) followed by dehydration in absolute ethanol for 24 h. The samples were mounted over stubs with gummy carbon tape followed by gold coating for 3 to 4 min with a vacuum ion sputter coater. The samples in three replicates were examined at different magnifications (20 kV) under a scanning electron microscope (H-7650; Hitachi).

Pathogenicity assay on detached leaves. The pathogenicity of *S. sclerotiorum* preexposed to *B. amyloliquefaciens* EZ1509 during antagonism assay was tested on detached leaves. A fungal mycelial plug of 0.6 cm was taken from the margin of the inhibition zone after exposing fungal mycelia to antagonist for 4 days and placed on one side of the detached leaves of rapeseed and tobacco. The leaves in control treatment were inoculated with a 0.6-cm block from 4-day-old fungal cultures without bacterial antagonist. The petioles were wrapped with cotton wools to maintain turgidity. The leaves were placed in square petri plates (10 × 10 cm) and incubated at 25°C with a 12-h photoperiod cycle. Pathogenicity of *S. sclerotiorum* was evaluated by measuring the lesion diameter. The experiment was repeated three times, with three replicates of each treatment in a completely randomized design.

Effect of *B. amyloliquefaciens* EZ1509 on oxalic acid production. To assess the production of oxalic acid, PDA plates were amended with bromophenol blue as an indicator, which emits blue color at pH ≥ 4.6 and yellow color at pH ≤ 4.6 (Steadman et al. 1994). To investigate the effect of *B. amyloliquefaciens* EZ1509 on the production of oxalic acid by *S. sclerotiorum* preexposed to bacterial antagonist during antagonism assay, mycelial plugs (0.6 cm) were taken from the fungal colony from the edge of inhibition zone and transferred to bromophenol blue-amended PDA plates. These plates were incubated at 25°C for 72 h. Oxalic acid production was recorded by measuring the diameter of the acidification zone (yellow color) after every 24 h in three replicates of each treatment, and the experiment was repeated three times.

RNA extraction and quantitative reverse transcription PCR analysis of LPs biosynthetic and virulence genes during antagonism assay. To confirm the expression of LP genes during coculturing, total RNA was extracted from three replicate plates on each day up to 4 days in dual-culture assay including *B. amyloliquefaciens* EZ1509 and fungus during antagonism. The RNA for control samples was extracted using the bacterial RNA kit (OMEGA) as per the manufacturer's guidelines from three replicate plates, on which bacteria were cultured without fungal pathogen. The expression of major genes involved in virulence of *S. sclerotiorum* was studied by extracting the RNA from fungal mycelium on the fourth day of dual-culture assay with *B. amyloliquefaciens* EZ1509. Total RNA was extracted from three replicates of dual-culture assay including *S. sclerotiorum* mycelia and *B. amyloliquefaciens* EZ1509 during antagonism at 96 h postinoculation, and the RNA for control samples was extracted from three replicate plates on which *S. sclerotiorum* was grown without bacterial antagonist by using Takara RNAiso Reagent (Takara Biotechnology Co.) as per the manufacturer's instructions. The concentration and purity of the RNA were determined by measuring the absorbance at 260/280 nm (NanoDrop 1000; Thermo Scientific). The complementary DNA (cDNA) first strand was synthesized using 5× All-In-One Rt MasterMix with the

AccuRT genomic DNA Removal Kit (ABM). The synthesized cDNA was used as a template for quantitative reverse transcription PCR (qRT-PCR).

qRT-PCR study of gene expression using SYBR Green.

The qRT-PCR analysis was carried out in a QuantStudio Real-Time Thermocycler (Thermo Fisher Scientific) using chamQ SYBR green qRT-PCR Master mix (Vayzyme) following the manufacturer's manual. Specific primers for the expression of LPs genes (fengycin, iturin, bacilysin, and surfactin) and virulence genes in *S. sclerotiorum* were designed to conduct qRT-PCR analysis (Table 3). The primers for iturin gene were designed using *B. amyloliquefaciens* CAU B946 iturin gene sequence as a reference, whereas *B. amyloliquefaciens* FZB42 genome was used for designing the primers of bacilysin, surfactin, and fengycin. The *RPSJ* and *actin* gene already reported as housekeeping genes were used as internal controls for *B. amyloliquefaciens* EZ1509 and *S. sclerotiorum*, respectively (Silva et al. 2009; Jordan et al. 2006). Quantitative real-time PCR was carried out in a 20-μl reaction volume containing 10 μl of 2× SYBR premix Ex Taq (Til RNaseH Plus) with 0.4 μl of Rox as a reference dye, 0.4 μl of forward and reverse primers (20 nmol), 2 μl of cDNA (100 ng), and 6.8 μl of double distilled water. The expression was recorded at the following conditions: (1) initial denaturation at 95°C for 30 s and (2) 40 cycles of 95°C for 10 s and 30 s at 60°C. The final relative expression of the target genes in three replicates was quantified using $\Delta\Delta C_t$ (Livak and Schmittgen 2001).

Statistical analysis. In vitro assays were performed in a completely randomized design. Data on in vitro antagonism assay, oxalic acid production test, detached leaf assay, and virulence gene expression were subjected to one-way analysis of variance (ANOVA), whereas LPs biosynthetic gene expression was subjected to two-way ANOVA using statistical package SPSS version 20.0. Treatment means were separated using Tukey's honestly significant difference at $P \leq 0.05$ after ANOVA and are expressed as means ± standard error of the mean.

TABLE 3. Oligonucleotides used in this study for quantitative reverse transcription PCR analysis

Oligo name	Sequence 5' to 3'
Iturin F	GAGTTCGCTTCAGCATCTT
Iturin R	AGTCTCCGTAGGCCCATATAC
Fengycin F	GTCGCAGAGCTTCAGAGAAA
Fengycin R	GATGGACCGTCAGAAACAAGTA
Bacilysin F	CTGTCCGAGATGTCACAAGAA
Bacilysin R	CGGCTTTTCAGGCGTTTAATATC
Surfactin F	GAACGCTATAAACGCGATGTG
Surfactin R	ATGATTGCTCCAGACGAGATAC
RPSJ F	GAAACGGCAAAACGTTCTGG
RPSJ R	GTGTGGGTTTACAATGTCG
SSPG 6 F	AAACAGCATGCACTGCTTCA
SSPG 6 R	TCAGCACAATTGCTGTGCGAG
SSPG 3 F	CGCACACAACAGTGATGGAT
SSPG 3 R	GAACAGCCATTCCGGTTAC
CBH F	ATCGGAGCTCGATCAACTGT
CBH R	GTCCAGCATCTGTCCGATA
ASPS F	CCACCAAATTCACCGTTGGT
ASPS R	TCGGCCAACATAAGAGTGGT
OAH F ^a	GCGCTCCAACAACCTTGGTTA
OAH R	CAGTCTTTGACGCCATCTCC
BXYL F	GCAGCTGGCTCGAATTACTC
BXYL R	GTGGATCAATGCGTCGTCAA
β-GLU F	GATCGGACCCGTGATTGTTG
β-GLU R	CGTCCGTGAGAGAATTTCCG
GLUCAN F	TGTGTGCCTTCCCATCTCTT
GLUCAN R	TGCAGACTGTAGCGTCATCCA
ACIDP F	TCCGCATACGTTGTCTACGA
ACIDP R	GCTGTTGCTGCATTGGAAAC
ACTIN F	CAACGATTGAGCGAGGATACA
ACTIN R	CGACAAGATGAGGTTGAAGAG

^a OAH, oxaloacetate hydrolase.

RESULTS

Screening for antagonistic activity. Dual-culture screening of five *Bacillus* strains revealed that *B. amyloliquefaciens* EZ1509 produced the largest inhibition zone against *S. sclerotiorum* followed by FZB42 and NMTD17 (Fig. 1). Strain LLCG23 showed the lowest inhibition, whereas strain 168 completely failed to inhibit the growth of the pathogen. Based on statistical analysis, all strains differed significantly from each other except for strain 168 and control. Based on dual-culture assay, *B. amyloliquefaciens* EZ1509 was used for additional antifungal assays against *S. sclerotiorum*.

PCR amplification of antifungal LPs biosynthetic genes in EZ1509. PCR amplification of LPs biosynthetic genes (viz. fengycin, surfactin, iturin, and bacilysin) in the genome of *B. amyloliquefaciens* EZ1509 indicated the presence of all LPs biosynthetic genes (Fig. 2).

Expression of antifungal LPs biosynthetic genes during antagonism. The relative expression of antifungal LPs biosynthetic genes (fengycin, iturin, bacilysin, and surfactin) is presented in Figure 3. Gene expression was studied on 4 consecutive days during dual-culture assay after every 24 h. The expression pattern of LPs genes in *B. amyloliquefaciens* EZ1509 varied from day 1 to day 4. All LPs biosynthetic genes showed the highest upregulation on the second day during antagonism assay, but the expression was significantly decreased on the third and fourth days. The expression of genes showed variation in a time-dependent manner. Iturin showed the highest expression compared with other LPs biosynthetic genes on the first and second days of interaction, whereas on the third and fourth days, fengycin showed the highest expression compared with control. Overall, expression of LPs genes was higher than control on all days during coculturing.

Characterization of LPs through MALDI-TOF-MS. MALDI-TOF-MS analysis of LPs extracts from solid PDA medium allowed characterization of LPs involved in antifungal activity of *B. amyloliquefaciens* EZ1509 against *S. sclerotiorum*. Mass spectra signals confirmed the presence of three major LPs families (viz. surfactin, fengycin, and iturin homologs) in both LPs extracts (Fig. 4). Two homologs of surfactin were detected at m/z 1,037.50 $[M + H]^+$ and 1,058.65 $[M + Na]^+$ from the ZCBC, whereas it was observed at 1,030.58 $[M + Na]^+$, 1,058.65 $[M + Na]^+$, and 1,088.65 $[M + K]^+$ from the ZCFC. Iturin was identified at three different masses (1,081.61 $[M + K]^+$, 1,109.56 $[M + K]^+$, and 1,123.58

$[M + K]^+$) in the ZCBC, whereas it was characterized at m/z 1,081.61 $[M + Na]^+$ and 1,102.67 $[M + H]^+$ in the ZCFC. Moreover, fengycin peaks were identified at 1,501.80 $[M + K]^+$ and 1,499.81 $[M + Na]^+$ in both the ZCBC and the ZCFC, respectively. However, bacilysin was not detected in both samples. LPs were identified by comparing their masses with previously reported masses of respective compounds in the literature (Table 4).

Ultrastructural changes in *S. sclerotiorum*. The effect of LP-producing *B. amyloliquefaciens* EZ1509 on the morphology of *S. sclerotiorum* was assessed during dual-culture assay. *B. amyloliquefaciens* EZ1509 caused ultrastructural abnormalities

Fig. 2. PCR amplification of antifungal lipopeptide genes in *Bacillus amyloliquefaciens* EZ1509. Bly, bacilysin; CK, control (without DNA); Fen, fengycin; Itu, iturin; Srf, surfactin.

Fig. 1. Screening of *Bacillus* strains for their antagonistic activity against *Sclerotinia sclerotiorum* in a dual-culture assay. **A**, Dual-culture assay. **B**, Mycelial growth inhibition zone diameter (centimeters) of *S. sclerotiorum* by five *Bacillus* strains. Means with similar letters are not statistically different from each other as analyzed by Tukey's honestly significant difference test at $P \leq 0.05$. Error bars represent standard error $\pm$ standard error of the mean.

in the mycelium of *S. sclerotiorum* that were observed through a scanning electron microscope after 4 days of incubation. SEM analysis indicated mycelial deformities, such as plasmolysis, disintegration, shrinkage, and breakdown of fungal hyphae, compared with control. In the control treatment, the mycelium was healthy, round, and cylindrical. The effect of *B. amyloliquefaciens* EZ1509 on *S. sclerotiorum* hyphae is presented in Figure 5.

Detached leaf assay. Pathogenicity of *S. sclerotiorum* was assessed in a detached leaf assay on tobacco and rapeseed. *S. sclerotiorum* previously exposed to *B. amyloliquefaciens* EZ1509 suppressed the lesions development on tobacco and rapeseed leaves (Fig. 6).

Oxalic acid production test. The effect of *B. amyloliquefaciens* EZ1509 on the production of oxalic acid by *S. sclerotiorum* was tested on PDA plates amended with bromophenol blue (Fig. 7). After 24 hours postinoculation (hpi), control (untreated) plates produced more yellow color compared with treated plates,

indicating the production of oxalic acid by the pathogen. After 72 hpi, control plates turned completely yellow. However, bacteria-treated *S. sclerotiorum* plates showed less depletion in blue color.

Expression of virulence genes in *S. sclerotiorum* by qRT-PCR. The relative expression of major virulence genes of *S. sclerotiorum* during in vitro interaction assay with *B. amyloliquefaciens* EZ1509 is shown in Figure 8. Results revealed that *B. amyloliquefaciens* EZ1509 downregulated the expression of all studied virulence genes. Among nine virulence genes, endopolygalacturonase 3 (SSPG3), oxaloacetate hydrolase (OAH), endopolygalacturonase 6 (SSPG6), and cellobiohydrolase were the highly affected genes compared with control.

DISCUSSION

Several members of the genus *Bacillus* are antagonistic to plant pathogens and can be used as potential biological control agents (Emmert and Handelsman 1999). *Bacillus* species use antibiosis as the primary mode of action to suppress fungal pathogens (Chowdhury et al. 2015; Zokaefar et al. 2014) and produce a diverse group of antimicrobial LPs that differ in mechanism of action, molecular mass, biochemical features, and genetic makeup (Velho et al. 2011). Their antagonistic effect is mainly attributed to the production of antifungal LPs (Susi et al. 2011). Dual-culture assay has been widely used to investigate direct antagonism of biological control agents (Arrebola et al. 2010; Farzand et al. 2019; Torres et al. 2016; Zhang et al. 2012). In this study, *B. amyloliquefaciens* EZ1509 emerged as the most promising antifungal strain among five *Bacillus* strains. In vitro suppression of radial growth of *S. sclerotiorum* has been reported previously (Rahman et al. 2016; Sharma and Sharma 2008; Souto et al. 2004; Yang et al. 2009). The formation of inhibition zones to suppress target pathogens was the result of excretion of antimicrobial compounds produced by *Bacillus* species (Kumar et al. 2012). Among antimicrobial compounds, antifungal LPs are regarded as the key components of *Bacillus* species involved in the direct

Fig. 3. Expression profile of potent lipopeptide biosynthetic genes in *Bacillus amyloliquefaciens* EZ1509 during coculturing. Means with similar letters are not statistically different from each other as analyzed by Tukey's honestly significant difference test at $P \leq 0.05$. Error bars represent standard error $\pm$ standard error of the mean.

Fig. 4. Matrix-assisted laser desorption/ionization-time of flight mass spectra of *Bacillus amyloliquefaciens* EZ1509 on positive ion mode. Lipopeptide extracts from **A**, the inhibition zone close to the bacterial colony and **B**, the inhibition zone close to the fungal colony near the inhibition zone.

antagonism of fungal pathogens (Cawoy et al. 2015). In this work, we have confirmed the presence of four antifungal LPs biosynthetic genes (viz. surfactin, fengycin, iturin, and bacilysin) producing genes in the genome of *B. amyloliquefaciens* EZ1509 through PCR amplification of respective genes. Previously, PCR detection methods have been used to confirm the presence of biosynthetic genes encoding surfactin, fengycin, iturin, bacilysin, and difficidin in the genus *Bacillus* (Rahman et al. 2016).

Bacillus species upregulate the expression of LPs biosynthetic genes during direct antagonism assay (Grabova et al. 2016; Sajitha and Dev 2016; Zihahirwa Kulimushi et al. 2017). It strengthens the fact that the genus *Bacillus* suppresses the growth of pathogens using LPs as a major weapon contributing to its inhibitory effect. In our previous study, we have reported the antifungal activity of LPs extracted from *B. amyloliquefaciens* EZ1509 against *S. sclerotiorum* (Farzand et al. 2019). LPs gene expression during antagonism assay involving *S. sclerotiorum* and *B. amyloliquefaciens* EZ1509 was studied by qRT-PCR on each day. All studied antifungal LPs biosynthetic genes were differentially expressed from day 1 to day 4. According to a report, surfactin, fengycin, and bacilysin biosynthetic genes showed differential expression during antagonism of *Bacillus subtilis* B1 against *Lasiodiplodia theobromae* (Sajitha and Dev 2016). In another instance, the expression of LPs genes encoding surfactin, fengycin, iturin, bacillomycin, bacilysin, and mycosubtilin was recorded through qRT-PCR in a dual-culture assay involving *B. subtilis* B1 and *L. theobromae* (Sajitha et al. 2016). In our study, fengycin and iturin genes were the most highly induced by the presence of fungus. Gene expression and subsequent synthesis of fengycin and iturin have been associated with antagonism of *Bacillus* strains (Grabova et al. 2016). We found that the bacilysin gene was expressed on the first day of antagonism assay, contrary to the study of Sajitha and Dev (2016) reporting that bacilysin gene was expressed on the fourth and fifth days of antagonism assay. In this study, surfactin gene was expressed at all days during coculturing, confirming earlier reports (Sajitha and Dev 2016). In an attempt to study the expression of LPs biosynthetic genes, Cawoy et al. (2014) reported the highest gene expression of fengycin on the fourth day of antagonism assay. However, in our study, fengycin showed highest expression on the second day of antagonism assay. The expression of genes kept decreasing on the third and fourth days. The decrease in gene expression might be because it is directly related to messenger RNA (mRNA) produced in bacteria, and the rapid fall in gene expression on later days is because of mRNA decay, which serves continuously to adjust the message according to the needs of cells for a specific protein (Condon 2003; Deutscher 2006).

Bacillus and *Pseudomonas* species have the potential to fine tune their strategy to produce different antibiotics on interaction with a particular pathogen (Garbeva et al. 2011; Li et al. 2014; Zhu et al. 2013). The production of antifungal LPs from whole-cell surface

extracts of *Bacillus* species has been previously reported through MALDI-TOF-MS (Athukorala et al. 2009; Vater et al. 2002). In this study, MALDI-TOF-MS confirmed the presence of surfactin, iturin, and fengycin in the inhibition zone. The production of LPs in growth medium by *B. subtilis* B1 in coculture with *L. theobromae* has been documented (Sajitha et al. 2016). Surfactin, fengycin, and iturin produced by *B. subtilis* were previously detected in the inhibition zone during dual-culture assay through ultraviolet-MALDI-TOF (Torres et al. 2016). Bacilysin was not detected in bacterial crude extract through MALDI-TOF-MS. This could be because of the fact that MALDI-TOF-MS is less sensitive to detection of smaller molecules (<500 Da); therefore, bacilysin, which is a low-molecular weight compound (271 Da), cannot be detected through MALDI-TOF-MS analysis (Pan et al. 2007).

Bacillus strains coproducing three LPs (surfactin, fengycin, and iturin) are globally the most antifungal strains (Cawoy et al. 2014), and the synergistic effect of LPs has been reported against several pathogens (Maget-Dana et al. 1992; Ongena et al. 2007; Romero et al. 2007). The antifungal effect of iturin is reported to be primarily based on pore formation in biological membranes that causes osmotic perturbation (Maget-Dana and Peypoux 1994). Iturin makes an association with biological membranes, anchors into lipid bilayers, generates irreversible pores by the insertion of surfactin molecules, and acts synergistically with surfactin to cause membrane disruption and solubilization (Ongena and Jacques 2008; Malfanova et al. 2012). Surfactin is the most commonly characterized LP, with excellent surface and membrane active properties and emulsifying and foaming effects (Vater et al. 2002). Surfactin-producing *Bacillus* strains exhibit high swarming motility and biofilm formation that ultimately facilitates bacterial cell motion. Fengycin has been reported to show high antifungal and antibacterial effect (Kim et al. 2004), particularly against filamentous fungi (Hofemeister et al. 2004; Vanittanakom et al. 1986). Fengycin interacts with lipid bilayers, and it causes

Fig. 5. Ultrastructural changes in *Sclerotinia sclerotiorum* mycelium under a scanning electron microscope. **A**, Treated. **B**, Control.

TABLE 4. Major peaks of lipopeptides produced by *Bacillus amyloliquefaciens* EZ1509 on solid media against *Sclerotinia sclerotiorum* characterized by Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry

Lipopeptide	Mode	Reported mass	Reference	Experimental mass	
				ZCBC ^a	ZCFC
Surfactin	[M + Na] ⁺	1,030.64	Dimkić et al. 2017	– ^b	1,030.58
Surfactin	[M + H] ⁺	1,036.69	Dimkić et al. 2017	1,037.50	–
Surfactin	[M + Na] ⁺	1,058.71	Torres et al. 2016	1,058.65	1,058.65
Surfactin	[M + K] ⁺	1,088.68	Torres et al. 2016	–	1,088.65
Iturin	[M + Na] ⁺	1,081.54	Torres et al. 2016	1,081.61	1,081.61
Iturin	[M + H] ⁺	1,102.51	Torres et al. 2016	–	1,102.67
Iturin	[M + K] ⁺	1,109.50	Pathak and Keharia 2014	1,109.5	–
Iturin	[M + K] ⁺	1,123.60	Price et al. 2007	1,123.58	–
Fengycin	[M + Na] ⁺	1,499.80	Dimkić et al. 2017	–	1,499.81
Fengycin	[M + K] ⁺	1,501.80	Vater et al. 2002	1,501.80	–

^a ZCBC, samples extracted from the inhibition zone close to the bacterial colony; ZCFC, samples extracted from the inhibition zone close to the fungal colony.
^b Dashes indicate mass not detected.

membrane solubilization, alters membrane structure and permeability, and creates ion-conducting channels in biological membranes (Deleu et al. 2005). Therefore, the coproduction of surfactin, fengycin, and iturin might be a cause of the strong antifungal activity of *B. amyloliquefaciens* EZ1509.

SEM revealed visible ultrastructural changes in *S. sclerotiorum* hyphae previously challenged with *B. amyloliquefaciens* EZ1509.

The ability of *B. amyloliquefaciens* EZ1509 to produce antifungal LPs could explain the observed results. It has been documented that antifungal LPs alter the ultrastructure of biological membranes, which leads to leakage of cellular metabolites and results in cell death (Brogden 2005). Similar ultrastructural changes in *S. sclerotiorum* hyphae during dual-culture interaction have been observed previously by Kamal et al. (2016). Sajitha and Dev

Fig. 6. Effect of *Bacillus amyloliquefaciens* EZ1509 on pathogenicity of *Sclerotinia sclerotiorum*. **A**, Pathogenicity assay on detached rapeseed and tobacco leaves. **B**, Effect of *B. amyloliquefaciens* EZ1509 on lesion diameter compared with untreated control. Means with similar letters are not statistically different from each other as analyzed by Tukey's honestly significant difference test at $P \leq 0.05$. Error bars represent standard error $\pm$ standard error of the mean.

Fig. 7. Effect of *Bacillus amyloliquefaciens* EZ1509 on acidification zone indicative of oxalic acid by *Sclerotinia sclerotiorum*. **A**, Plate assay amended with bromophenol blue. **B**, Diameter of acidification zone indicating oxalic acid production under influence of *B. amyloliquefaciens* EZ1509 at different time intervals in hours postinoculation (hpi) compared with untreated control. Means with similar letters are not statistically different from each other as analyzed by Tukey's honestly significant difference test at $P \leq 0.05$. Error bars represent standard error $\pm$ standard error of the mean.

(2016) observed plasmolysis and shrinkage of fungal hyphae of *L. theobromae* in the inhibition zone during antagonism assay owing to antifungal LPs produced by *B. subtilis* B1.

Oxalic acid is considered as a primary determinant of pathogenicity of *S. sclerotiorum* (Bolton et al. 2006; Godoy et al. 1990). *S. sclerotiorum* initiates the production of oxalic acid to create a conducive environment for the release of hydrolytic enzymes. We hypothesized that *B. amyloliquefaciens* EZ1509 might influence oxalic acid production by *S. sclerotiorum* as a result of antagonistic interaction. Bromophenol blue test indicated reduced acidification, which is indicative of reduced oxalic acid production by *S. sclerotiorum* challenged with *B. amyloliquefaciens* EZ1509 compared with control. The inhibitory activity of *B. amyloliquefaciens* EZ1509 on pathogenicity of *S. sclerotiorum* is an indication of weak compatibility between *Bacillus*-treated pathogen and host tissues. The weak interaction between host and pathogen might be owing to the inability of *S. sclerotiorum* to produce oxalic acid. Massawe et al. (2018) reported that volatile organic compounds (VOCs) emitted by *Bacillus* species altered the ability of *S. sclerotiorum* to release oxalic acid and reduced the pathogenicity level to the assayed host plants. However, unlike their study, we have associated the reduced pathogenicity of *S. sclerotiorum* with the effect of diffusible antifungal LPs producing *B. amyloliquefaciens* instead of VOCs.

S. sclerotiorum contains a plethora of virulence-related genes that are directly involved in pathogenicity. During host penetration and subsequent infection stages, the fungus must break the layers of host defense barriers. To achieve this goal, the fungus produces a cocktail of hydrolytic enzymes, effector proteins, and secondary metabolites. OAH is a key hydrolytic enzyme that converts oxaloacetate to oxalic acid. Oxalic acid is secreted in early events of fungal infection, and it is crucial for the suppression of host defense (Moosa et al. 2018; Williams et al. 2011). Disruption of the OAH gene in *S. sclerotiorum* leads to reduced virulence (Liang et al. 2015). Genes encoding acid protease and aspartyl protease in *S. sclerotiorum* are involved in the decomposition of host defense proteins at early stages of infection (Poussereau et al. 2001) and cell death (Movahedi and Heale 1990). Endopolygalacturonase enzymes cause degradation of cell wall pectin and lysis of fungal hyphae (Kazemi-Pour et al. 2004; Moosa et al. 2018; Raymond et al. 1994). Hyphal deformities, inability to produce oxalic acid, and reduced pathogenicity of *S. sclerotiorum* provoked us to study the effect of bacterial antagonist on pathogenicity-related genes of the fungal pathogen. The results revealed that the expression of virulence genes was significantly reduced in fungal mycelium previously challenged with *B. amyloliquefaciens* EZ1509 in dual culture. To the best of our knowledge, current study is the first report of reduced expression of virulence-linked genes in

S. sclerotiorum challenged with the bacterial antagonist in a dual culture assay. Previously, the VOCs produced by the genus *Bacillus* altered the expression of several virulence genes of *Ralstonia solanacearum* and reduced the incidence of wilt disease (Tahir et al. 2017).

Bacteria use different mechanisms to control fungal pathogens, but we hypothesized that diffusible LPs are the main antifungal weapon of *B. amyloliquefaciens* EZ1509 during direct antifungal activity against *S. sclerotiorum*. Mutants of *B. amyloliquefaciens* C06 and *B. amyloliquefaciens* FZB42 deficient in one or more LPs biosynthetic genes lost their antifungal activity against fungal pathogens in dual-culture assay (Cawoy et al. 2014; Gu et al. 2017; Liu et al. 2011). In a comparative study with six *B. subtilis*/*B. amyloliquefaciens* strains, the antifungal activity of the strains was found to be connected with their ability to synthesize cyclic LPs (Chowdhury et al. 2015). Moreover, an increase in the production of fengycin and iturin by *B. amyloliquefaciens* has been observed in the presence of certain plant pathogens (Cawoy et al. 2015).

Conclusively, *B. amyloliquefaciens* EZ1509 can perceive specific elicitors molecules emitted by the pathogen, and it can upregulate the biosynthesis of LPs. Our study shows that the bacterium produces different LPs (fengycin, iturin, surfactin, and bacilysin) during cocultivation with the fungus, and this cocultivation led to deteriorations in fungal mycelium, reduced pathogenicity, and downregulation of virulence genes on coculturing. Hence, we infer the conclusion that *B. amyloliquefaciens* EZ1509 can be considered as a very effective biocontrol agent to minimize the destructive effects of *S. sclerotiorum* on host plants.

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Fig. 8. Effect of *Bacillus amyloliquefaciens* EZ1509 on expression of virulence genes of *Sclerotinia sclerotiorum*. Means with similar letters are not statistically different from each other as analyzed by Tukey's honestly significant difference test at $P \leq 0.05$. Error bars represent standard error $\pm$ standard error of the mean.

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