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ORIGINAL ARTICLE

Bacillus amyloliquefaciens alters the diversity of volatile and non-volatile metabolites and induces the expression of defence genes for the management of Botrytis leaf blight of *Lilium* under protected conditions

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Abstract
Lilium leaf blight induced by *Botrytis cinerea* is a major threat in the cultivation of *Lilium* in the Nilgiris province of Tamil Nadu, India under protected conditions. The present study aims to understand bipartite and tripartite interactions between *B. cinerea*, *Lilium* and *B. amyloliquefaciens* to develop a cost-effective antagonist to manage Botrytis leaf blight. The in vitro antagonism by *B. amyloliquefaciens* (VB7) suppressed the mycelial growth of *B. cinerea* by up to 46%. Foliar application of *B. amyloliquefaciens* (VB7) colonized the phylloplane within 48 h and prevented conidial germination of *B. cinerea*. Biofilm on the leaf surface colonized the conidia of *B. cinerea* (SEL). The colonized conidia were parasitized by bacterial cells, resulting in shrinkage. The bipartite interaction between *B. amyloliquefaciens* (VB7) and mycelial biomass of *B. cinerea* as sole carbon source produced volatile and non-volatile antifungal compounds. Tripartite interactions between *Lilium* leaf, *B. amyloliquefaciens* (VB7) and conidia of *B. cinerea* produced five different non-volatile antifungal and antibacterial compounds. Expression of defence genes through qRT-PCR analysis indicated that the transcript level of *PAL*, *PR 10* and ascorbate peroxidase (APX) were triggered in *Lilium* plants treated with *B. amyloliquefaciens* (VB7), challenged against *B. cinerea* (SEL). Bulb dipping and soil drenching along with foliar application suppressed *Botrytis* leaf blight and increased *Lilium* stem yield under protected conditions.

Keywords Leaf blight of Lilium · *Bacillus* · Antifungal metabolites · ESEM · Defence genes

Introduction

Lilium cut flowers are cultivated for their scented nature and attractive colours. *Lilium* comprises around 100 species distributed throughout the temperate regions across the globe (Siljak-Yakovlev et al. 2003). In the northern parts of India, it is cultivated in Himachal Pradesh and Uttaranchal. In the southern part, it is cultivated in the Nilgiris province of Tamil Nadu. The crop is infected by leaf blight pathogens caused by *Botrytis cinerea*, an emerging disease in India (Dhyani et al. 2012). *Botrytis* leaf blight destroyed 15% of the plants under greenhouse condition and caused economic loss in *Lilium* production (Hou and Chen 2003; Cao et al. 2018). *Botrytis cinerea* causes foliage blight and in severe stages, it affects the floral parts (Dhyani et al. 2012; Priyanka and Nakkeeran 2018). *Botrytis* species can be managed by proper cultural measures like weed control, removal of infected parts, and good air circulation to reduce humidity and fungicide applications. Though synthetic fungicides are cheaper and effective for *Botrytis* blight management, application of fungicides causes detrimental effects not only on the pathogen but also on beneficial microbes. In addition, the repeated use of fungicides might result in resistance development among fungal pathogens and cause environmental pollution. To overcome the issue, plant-growth promoting rhizobacteria (PGPR) can serve as a substitute for the management of *Botrytis* blight of *Lilium*.

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In recent years, PGPR have been used in disease management. The rhizosphere soil is bestowed with effective PGPR with different modes of action including the production of plant hormones, nutrient mobilization, production of antibiotics and hydrolytic enzymes, antimicrobial compounds, induction of host defence and hence is used for the management of plant pathogens (Wu et al. 2018). Several volatile and non-volatile compounds produced by different *Bacillus* species with antifungal nature were effective in suppressing several plant pathogens (Weisskopf 2013; Lim et al. 2017; Ji 2013). In addition, induced host plant resistance by PGPR through the activation of both SA and JA/ET signalling pathways has been reported against several fungal pathogens (Mishra et al. 2017). Specifically, microbial non-self-molecules are known to induce immunity in plants via the activation of signalling cascades resulting in coordinated expression of a specific set of genes involved in plant defence. Apart from disease suppression, VOC compounds induced in plants also have synergistic action with other metabolites and plant hormones promote plant growth and suppress plant pathogens (Dani et al. 2016; Brilli et al. 2019). The triggered immunity was analysed by quantifying the transcripts of different genes involved in the SAR and ISR pathways as well as in co-expression of defence compounds (Huckelhoven 2007). Likewise, *B. subtilis* GB03 and *B. amyloliquefaciens* as foliar spray induced defence signalling pathways through the production of volatile compound 2,3-butanediol in *Arabidopsis* (Ryu et al. 2003). Attempts were made to manage grey mould under protected conditions by characterizing volatile and non-volatile organic compounds (VOCs/NVOCs) from antagonistic *B. amyloliquefaciens* (VB7), assessing the ability of antagonistic *Bacillus* spp. to induce defence genes and evaluating the efficacy of *B. amyloliquefaciens* (VB7) under protected conditions.

Materials and methods

Isolation and characterization of *Botrytis cinerea*

The pathogen *B. cinerea* was isolated from infected leaves of 15 different *Lilium* varieties/hybrids (Beau soleil, Acapulco, Primium Blond, Siberia, Severan, Arlington, Selverio, Joop, Sorbone, Black out, Beah, Honesty, Eremo, Brindishi, and Nashville) collected from the Nilgiris district of Tamil Nadu province, India using standard protocols (Tutte 1969). Further, the pathogen was characterized through PCR using specific primers of *B. cinerea*: C729± 5′-AGCT CGAGAGAGATCTCTGA-3′ and 5′-CTGCAATGTTCT GCGTGGAA-3′ corresponding to the 18 s rRNA region (Rigotti et al. 2002). The genome product was amplified by initially denaturing at 94 °C for 5 min. It was followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C, extension for 1 min at 72 °C with a final extension for 10 min at 72 °C. The amplicon was analyzed in 1.2% agarose gel, amended with ethidium bromide, and viewed on a transilluminator. The amplicons were sequenced and confirmed for their identity in NCBI database. The pathogenicity of all the isolates was proved and the virulent isolate was used for further study.

Antifungal efficacy of *Bacillus* spp. against *B. cinerea* in vitro

The antifungal activity of 10 *Bacillus* spp. and one *Ochrobactrum intermedium* isolated from the rhizosphere of *Lilium* plants was tested against *B. cinerea* isolate Selverio (SEL) by the dual plate technique in PDA medium. A 5 mm mycelial disc of *B. cinerea* was placed 1 cm away from the margin on one side of the Petri plate and on the opposite side 24-h-old bacterial antagonists were streaked and incubated at 15 ± 2 °C. After incubating for 7 days, the radial growth of pathogen mycelium was recorded and inhibition per cent was calculated for the test pathogen.

GC-MS analysis of volatile and non-volatile organic compounds

Bacillus amyloliquefaciens (VB7) was cultured on both the heat-killed mycelial biomass of *B. cinerea* and without the mycelial biomass of *B. cinerea* and diversity of VOCs/NVOCs was analysed through GC-MS. *B. cinerea* (SEL) was grown for 7 days in potato dextrose broth. The mycelial biomass was filtered using a filter paper (Whatman No. 1) in a glass funnel. The mycelial mat was dried by incubating in a hot air oven for 48 h at 40 °C and homogenized with liquid nitrogen. To analyse the headspace, isolate VB7 was multiplied in Cephadex broth for 7 days at 15 °C. The VOC produced during the interaction of *B. amyloliquefaciens* (VB7) with the heat-killed mycelial biomass of *B. cinerea* at 10 g/100 ml as sole carbon source in the Cephadex broth at 15 °C for 7 days was trapped and analysed as described by Lee et al. (2015). Similarly, the VOC produced by *B. amyloliquefaciens* (VB7) multiplied in Cephadex broth for 7 days at 15 °C without the heat-killed mycelial mat of *B. cinerea* (SEL) was also analysed. NVOCs of *B. amyloliquefaciens* (VB7) and *B. amyloliquefaciens* (VB7) cultured on the heat-killed mycelial biomass of *B. cinerea* (SEL) as sole carbon (10 g/100 ml of Cephadex broth) were also analysed. The culture filtrates were centrifuged at 5000 rpm for 15 min at 4 °C. After discarding bacterial cells, the pH of the supernatant was adjusted to 2.0 with 1 N HCl. The concentrated, crude secondary metabolites extracted from culture broth of *B. amyloliquefaciens* (VB7) were used for GC/MS analysis (Dheepa et al. 2016).

Characterization of NVOCs produced during bipartite and tripartite interaction of *B. amyloliquefaciens* VB7 and *B. cinerea* (SEL) in *Lilium* plants through GC-MS analysis

This experiment was conducted to understand the change in the metabolite profile of *Lilium* plants upon treatment with (1) *B. cinerea* alone, (2) *B. amyloliquefaciens* alone and (3) both. Forty-eight-hour-old bacterial suspension of *B. amyloliquefaciens* VB7 was sprayed on to the leaves with an atomizer at 1% (3×10^8 cfu/ml). One day later, the leaves were challenged by foliar application with the conidial suspension of *B. cinerea* (4×10^6 cfu/ml) and maintained in the growth chamber at 15 °C, 90% RH for 72 h. Similarly, pathogen-inoculated control and healthy control were also maintained. For each treatment, seven replications were maintained with three plants per replication. The samples were collected and frozen immediately by immersing in liquid nitrogen. Lyophilized leaf samples were stored at −80 °C, processed and analysed. Five grams of lyophilized samples was transferred into a conical flask and 50 ml of ethyl acetate was added. The mixture was incubated overnight in a shaker at 37 °C. The solvent fraction was filtered with muslin cloth and condensed through a vacuum flask rotary evaporator. The condensate was air dried and then dissolved in HPLC grade methanol and filtered through a 0.2 μm syringe filter. Finally, the samples were analysed through GC-MS and compounds were identified by GC-MS using an Elite-5MS column (100% dimethylpolysiloxane), 30 × 0.25 mm × 0.25 μm df equipped with a Clarus 500 GC/MS (Perkin Elmer). The column temperature was maintained initially at 110 °C at the rate of 10 °C/min-No hold followed by increase up to 280 °C. Based on the NIST MS data library and spectrum obtained through GC/MS, the compounds present in the sample were identified and their diversity was compared using R studio software to study the abundance between treatments (Vinodkumar et al. 2017).

Studies on the interaction of *B. amyloliquefaciens* VB7 on the phylloplane of *Lilium*

B. amyloliquefaciens VB7 was used to determine antifungal activity and its efficacy was assessed against *Botrytis* leaf blight under protected conditions. A 48-h-old bacterial suspension of *B. amyloliquefaciens* VB7 at 1% (3×10^8 cfu/ml) was sprayed onto leaves with an automizer. One day later, the leaves were challenged by foliar application of conidial suspension of *B. cinerea* (4×10^6 cfu/ml) and maintained in the growth chamber at 15 °C, 90% RH for 72 h. Similarly, pathogen-inoculated control and healthy control were also maintained. For each treatment, three *Lilium* (Beau Soleil) plants were used and replicated seven times. Leaf samples were collected randomly from each treatment. From the collected samples, 1 to 2 cm samples were treated with 2% glutaraldehyde solution for 4 h and later they were treated with 30, 50, 70 and 100% acetone serially at 10 min intervals for each treatment. The samples were then serially rinsed with 30, 50, 70 and 100% ethanol at 10 min intervals. Finally, the samples were dried using a vacuum desiccator and observed under SEM (Quanta 250, FEI, Hillsboro, OR, USA). The SEM was operated at 2 kV vacuum, with a spot size of 3.5 and 60 Pa pressure. Observations on germination of *B. cinerea* (SEL) conidia, colonization of *B. amyloliquefaciens* VB7 around *B. cinerea* (SEL) conidia, germicidal action of the bacterial antagonist and biofilm formation were recorded after 48 h of interaction.

qRT-PCR analysis of defence genes

The expression profile of defence genes in *Lilium* plants treated separately with *B. amyloliquefaciens* (VB7), *B. cinerea* (SEL), and the plants treated with *B. amyloliquefaciens* (VB7) challenge inoculated with *B. cinerea* (SEL) were analysed for the defence gene expression using qRT-PCR. The healthy plants uninoculated with *B. amyloliquefaciens* (VB7) or *B. cinerea* (SEL) were maintained as control. Each treatment was replicated thrice. The RNA was isolated from the samples after 24 and 72 h inoculation (Onate-Sanchez and Vicente-Carbojosa, 2008). The cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). qRT-PCR was performed with tubulin as internal control to normalize defence gene expression in a Bio-Rad CFX 96 real-time PCR system with SYBR Premix Ex Taq II (Takara Clontech, Japan). The primers were synthesized according to Rao et al. (2014). The qRT-PCR analyses were performed by exposing the samples for 5 min at 95 °C, followed by 50 cycles for 20 s at 95 °C, 30 s at 63.5 °C and at 72 °C for 30 s.

Evaluating the bioefficacy of *B. amyloliquefaciens* VB7 against *Botrytis* blight under protected conditions

To assess the efficacy of *B. amyloliquefaciens* (VB7 and MM12), *B. subtilis* (GB, MM19 and VB2) and *O. intermedium* (MM13) against *Lilium* leaf blight, experiments were conducted during 2015–2016 and 2016–2017 under protected conditions. The bacterial suspension of different bacterial strains was prepared as per Vinodkumar et al. (2017). *Lilium* bulbs of Beau soleil variety were planted on raised beds of 30 cm height, 914 cm long and 91 cm wide with 10/10 cm spacing per replication. The bulbs were soaked for 30 min in 1% antagonistic bacterial suspensions (3×10^8 cfu/ml). Subsequently, the soil was drenched with the same suspension. The procedure was repeated at monthly intervals until flower bud initiation to suppress soil-borne

inoculum. The plants were also sprayed with the above-mentioned suspension at weekly intervals for 4 weeks. Treatment of bulbs, drenching on to soil and foliar application of *Pseudomonas fluorescens* (Pf1) at 3×10^8 cfu/ml was maintained as comparative check. An untreated control was also maintained. All the treatments were replicated thrice. From each treatment, 50 plants were randomly selected and tagged to record observations on the incidence of leaf blight, stem yield and stem height per m^2. Leaf blight incidence was calculated and expressed as per cent disease index (PDI) on a 0–5 scale (Datar and Mayee 1985).

Statistical analysis

Completely randomized block design (CRD) was performed in vitro for statistical analysis and randomized block design (RBD) was done for field experiments. The required number of replications was maintained for each experiment. Mean differences of the treatments were evaluated with ANOVA at 5% significance (Gomez and Gomez 1984). Data were analysed statistically with IRRISTAT (version 3/93, Biometrics Unit, International Rice Research Institute) and interpreted.

Results

Isolation and identification of pathogen

The pathogen isolated from blight-infected plants of 15 different *Lilium* varieties initially produced a hyaline mycelium that later turned grey. Dark black sclerotial bodies were produced after 15–30 days of incubation. PCR was performed using the specific primer pair C729± for *B. cinerea*, which yielded a 720 bp amplicon, suggesting all the isolates as *B. cinerea*. The nucleotide sequences of the amplicons of different isolates of *B. cinerea* were assigned with the accession numbers KU936079, KU936080, KU936081, KU936082, KU936083, KU936084, KU936085, KY490053, KY490054, KY490055, KY490056, KY490057, KY490058, KY490059, KY490060 and were deposited in the NCBI database.

Molecular characterization and in vitro screening of antagonistic *Bacillus* spp.

Eleven isolates of bacterial antagonists isolated from rhizosphere were amplified with a product size of 1500 bp corresponding to 16S rRNA and sequence determined. Sequence homology search using NCBI BLAST revealed that out of 11 isolates, four were *B. amyloliquefaciens* (KJ603230, MG645177, JX036522 and MH348121), two were *B. subtilis* ssp. *spizizenii* (MG645182 and MG645183) and one each from *B. cereus* (JX036520), *B. licheniformis* (KC540818), *B. subtilis* (JN873300) and *O. intermedium* (MG645186). Upon screening the 11 isolates against *B. cinerea*, seven isolates were found to inhibit the mycelial growth of *B. cinerea*, with a maximum of 46.30% by *B. amyloliquefaciens* (VB7) followed by the *B. subtilis* isolate VB3 (45.93%) (Supplementary Fig. 1).

Characterization of VOC/NVOC compounds by GC-MS analysis

GC-MS analysis of VOC and NVOC compounds revealed that 1,3-methanopentalene, 3,5-octadiyne (antimicrobial), *N*-ethyl-hexahydro-1H-azepine, cyclopentasiloxane, oxirane (antimicrobial), decane (antifungal and antibacterial) and formic acid had the maximum Row-Z-Score of >0.5 in *B. amyloliquefaciens* VB7 co-cultured in the presence of heat-killed mycelial biomass of *B. cinerea* (SEL). But, it was negatively regulated (Row-Z-Score = − 0.5) when *B. amyloliquefaciens* VB7 was cultured in the absence of heat-killed mycelial biomass of *B. cinerea* (Supplementary Figs. 2 and 3, Supplementary Tables 1 and 2).

Similarly, a comparative study on the diversity of NVOCs produced by *B. amyloliquefaciens* VB7 co-cultured in the presence of heat-killed mycelial biomass of *B. cinerea* (SEL) indicated that 1,2-benzenedicarboxylic acid (antimicrobial), 5-hydroxymethylfurfural (antimicrobial), propanoic acid (antifungal), hexadecanoic acid (antifungal), butyl 2-pentyl ester (antifungal) and phthalic acid (antifungal) was produced to the maximum level with the maximum Row-Z-Score of >0.5. However, these compounds were negatively regulated (Row-2-Score = − 0.5) when *B. amyloliquefaciens* VB7 was cultured in the absence of heat-killed mycelial biomass of *B. cinerea*. Likewise, NVOC compounds including 1,2,3-propanetriol, a methyl-I-sorboside, dihydroxyacetone, phthalic acid and dibutyl phthalate with maximum Row-Z-Score of >0.5 were observed only in *B. amyloliquefaciens* VB7 cultured in the absence of heat-killed mycelial biomass of *B. cinerea*, while the case was the reverse in *B. amyloliquefaciens* VB7 co-cultured in the presence of heat-killed mycelial biomass of *B. cinerea* (Supplementary Figs. 4 and 5, Supplementary Tables 3 and 4).

Characterization of NVOCs produced during bipartite and tripartite interactions of *B. amyloliquefaciens* VB7 and *B. cinerea* (SEL) in *Lilium* plants through GC-MS analysis

GC-MS analysis revealed that the uninoculated *Lilium* plant produced compounds like phthalic acid, 5-methylhex-2-yl isobutyl ester; 1,4-benzenedicarboxylic acid, bis(2-methylpropyl) ester; pentadecanoic acid; terephthalic acid, isobutyl butyl ester; phthalic acid, hepr-3-yl isobutyl ester; phthalic acid, non-5-yn-3-yl 2-pentyl ester; phytyl palmitate

and diisooctyl phthalate (Supplementary Fig. 6A, Supplementary Table 5). NVOCs detected in the bipartite interaction of *Lilium* plants with *B. amyloliquefaciens* (VB7) were 1,4-benzenedicarboxylic acid, bis(2-methylpropyl) ester; phthalic acid isobutyl 2-pentyl ester; dibutyl phthalate; n-hexadecanoic acid, phthalic acid, 5-methyhey-2-yl isobutyl ester and bis(2-ethylhexyl) phthalate (Supplementary Fig. 6B, Supplementary Table 6).

Similarly, bipartite interaction with the virulent isolate of *B. cinerea* (SEL) revealed the presence of phthalic acid, 5-methylhex-2-yl isobutyl ester; phthalic acid, hex-3-yl isobutyl ester; dibutyl phthalate; n-hexadecanoic acid; phthalic acid, butyl hept-3-yl ester and trisiloxane, octamethyl- (Supplementary Fig. 7A, Supplementary Table 7). Tripartite interaction of *Lilium* plants pre-immunized with *B. amyloliquefaciens* VB7 followed by challenge inoculation with *B. cinerea* produced metabolites including ethyl 3-nitrobenzoate; 1,4-benzenedicarboxylic acid, bis(2-methylpropyl) ester; phthalic acid, 5-methylhex-2-yl isobutyl ester; 1,4-benzenedicarboxylic acid, bis(2-methylpropyl) ester; n-hexadecanoic acid; phthalic acid, 5-methylhex-2-yl isobutyl ester; benzoic acid, 3-formyl-5-methyl-, trimethylsilyl ester; trimethylsilyl peroxide and trisiloxane, octamethyl- (Supplementary Fig. 7B, Supplementary Table 8). Comparison of the variation in the diversity of NVOCs produced during tritrophic interaction of *B. amyloliquefaciens* VB7 along with *Lilium* plant and *B. cinerea* conidia induced the expression of benzoic acid, trimethylsilyl peroxide and ethyl 3-nitrobenzoate with Row - Z-Score value of 1.5. However, these compounds were not produced in ditrophic interactions between *Lilium* plant and *B. cinerea* conidia or *Lilium* plant with *B. amyloliquefaciens* VB7 (Supplementary Fig. 8).

Environmental scanning electron microscopic studies on the interaction of *B. amyloliquefaciens* VB7 on *Lilium* phylloplane

Examination of *Lilium* leaves sprayed with *B. amyloliquefaciens* VB7 challenged with *B. cinerea* (SEL) revealed that the bacterial antagonist colonized *Lilium* phylloplane within 48 h after application (Fig. 1a). It was followed by the formation of biofilm (Fig. 1b). The conidia of *B. cinerea* (SEL) were surrounded and ramified by the bacterial cells of *B. amyloliquefaciens* VB7 (Fig. 1c). Subsequently the conidia were parasitized by the bacterial cells (Fig. 1e). Besides, shrinkage of *B. cinerea* (SEL) conidia was also noticed (Fig. 1d). However, in leaves sprayed with the conidia of *B. cinerea* (SEL) alone, conidial germination was observed (Fig. 1f) within 48 h. Thus, the electron microscopic observations confirmed the colonization, biofilm formation and ramification around *B. cinerea* (SEL) conidia, followed by the shrinkage of the conidia and germicidal action.

qRT-PCR analysis of defence genes

The effect of *B. amyloliquefaciens* (VB7) to trigger the expression of ascorbate peroxidase (APX), PAL and PR10 genes challenged with *B. cinerea* (SEL) was assessed at periodical intervals through qRT-PCR. The transcripts of PAL genes were downregulated in plant samples treated with *B. cinerea* (SEL) to 0.88-fold. However, it was upregulated to 17.94-fold in plants sprayed with *B. amyloliquefaciens* (VB7) challenged with *B. cinerea* (SEL) after 24 h. However, the expression of the PAL gene declined in plants sprayed with *B. amyloliquefaciens* (VB7) challenged with *B. cinerea* (SEL) after 72 h of inoculation (Fig. 2). Similarly, induction of PR10 genes indicated a 1.41-fold transcript level in the pathogen-inoculated control, whereas the same increased up to 2.48-fold after 24 h in *Lilium* plants sprayed with *B. amyloliquefaciens* (VB7) challenged against *B. cinerea* (SEL). However, after 72 h, the transcript level of PR10 genes treated with *B. amyloliquefaciens* (VB7) challenged against *B. cinerea* (SEL) increased up to 4.72-fold than pathogen-inoculated control plants (Fig. 2).

We observed a 27.01-fold increase in APX transcripts in *B. amyloliquefaciens* (VB7)-treated plants. Plants treated with *B. amyloliquefaciens* (VB7) challenged against *B. cinerea* (SEL) expressed a 48.57-fold increase in transcript level after 72 h of inoculation. However, there was no significant difference in the expression level of defence genes irrespective of the treatments after 24 h. The same trend was also observed after 72 h in plants treated with *B. cinerea* (SEL) alone when compared with the untreated control (Fig. 2).

Effect of bacterial antagonists on growth promotion and leaf blight management

Six different bacterial antagonists were assessed in vitro for the management of *Botrytis* blight of *Lilium*. Results revealed that bulb dipping, drenching of soil combined with foliar spray with 1% of *B. amyloliquefaciens* (VB7) (10 ml/L at 10^8 CFU/ml) had the minimum leaf blight of 11.71 PDI with a stem yield of 41 stems/m^2. Besides, it also increased the stem height to 108 cm as against 95 cm in the untreated control. It differed significantly from *B. amyloliquefaciens* VB2-treated *Lilium* plants which had 17.54 PDI, with a stem yield of 38 stems/m^2. However, the mean leaf blight incidence of 52.50 PDI was recorded in the untreated control with a mean yield of a 27 stems/m^2 (Fig. 3).

Discussion

Botrytis cinerea is a pervasive fungus causing severe damage during the vegetative growth phase and flowering stage of *Lilium*. Enhancing host plant resistance is one of the control

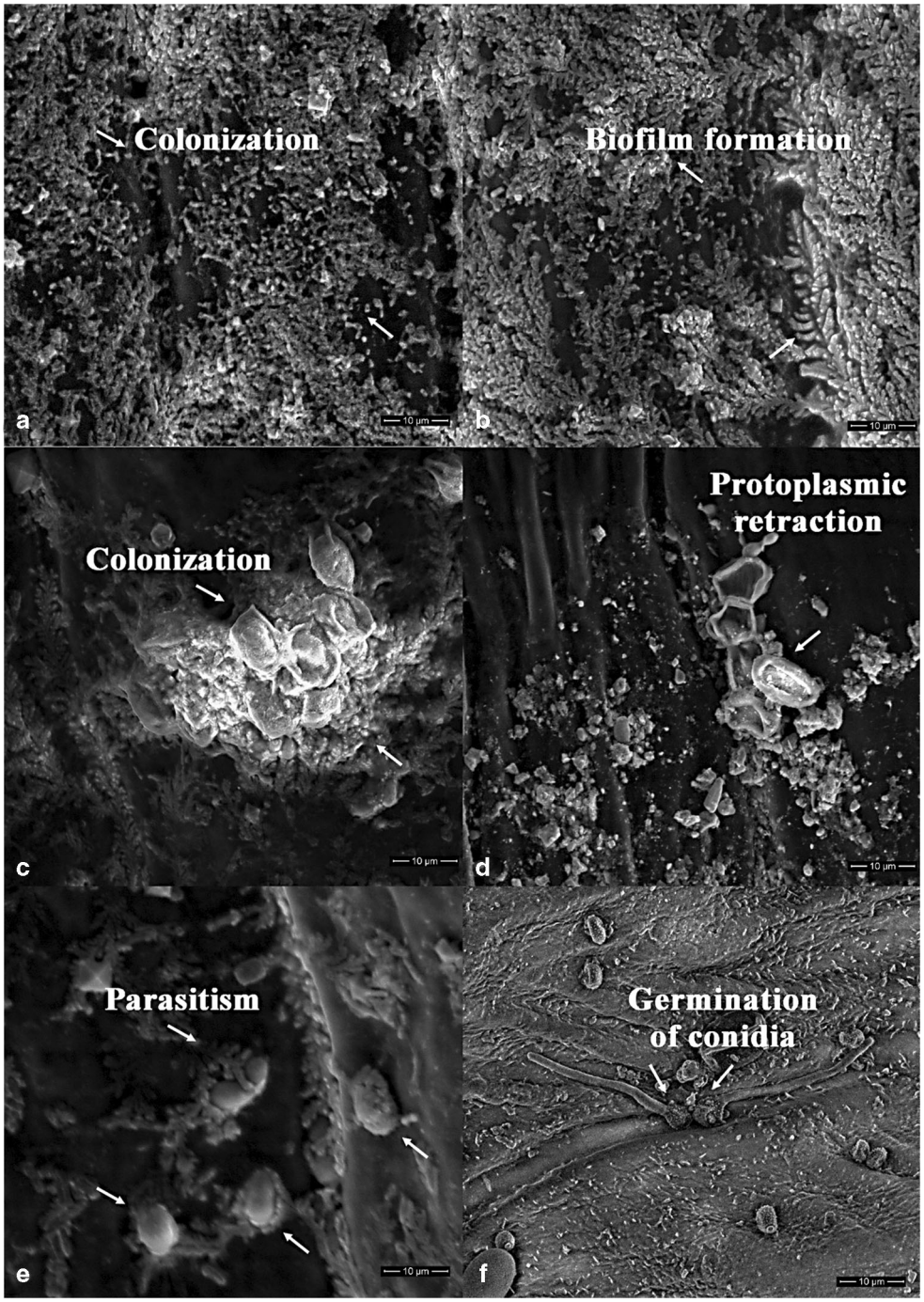

Fig. 1 Scanning electron microscopic observation. a Colonization of *Bacillus amyloliquefaciens* VB7 on the phylloplane of *Lilium.* **b** Biofilm formation by *B. amyloliquefaciens VB*7 on the phylloplane of *Lilium.* **c** Colonization by *B. amyloliquefaciens* VB7 over the conidial surface of *Botrytis cinerea* SEL. **d** Protoplasmic retraction of *B. cinerea* SEL conidia. **e** Parasitism by the bacterial cells of *B. amyloliquefaciens* VB7 on the conidia of *B. cinerea* SEL. **f** Germination of *B. cinerea* SEL conidia

measures to manage the disease as the disease-causing organism is a necrotrophic pathogen and has a wider host range. In our study, the isolated fungal pathogen was confirmed as *B. cinerea* through morphological characteristics and sequence determination by PCR. Similarly, Dhyani et al. (2012) confirmed the necrotrophic pathogen *B. cinerea* as a causative agent of flower and shoot blight in *Lilium polyphyllum.* Rigotti et al. (2002) also identified *B. cinerea* as the causative agent of *Lilium* blight (18SrDNA sequence size of 700 bp and having 98% homology with other isolates). Similarly, Mirzaei et al. (2007) also amplified the PCR product size of 700 bp from DNA isolated from *B. cinerea* isolates. Leaf blight of *Lilium* can be managed through the effective use of fungicides, although ecological problems abound. Hence, PGPR have been explored for the management of host plant resistance. In the present study, screening of 11 isolates of bacterial antagonists against mycelial growth of *B. cinerea* revealed the maximum inhibitory activity in *B. amyloliquefaciens* strain (VB7). Further, GC-MS analysis of secondary metabolites of *B. amyloliquefaciens* strain (VB7) exhibited different VOC and NVOC compounds. Kim et al.

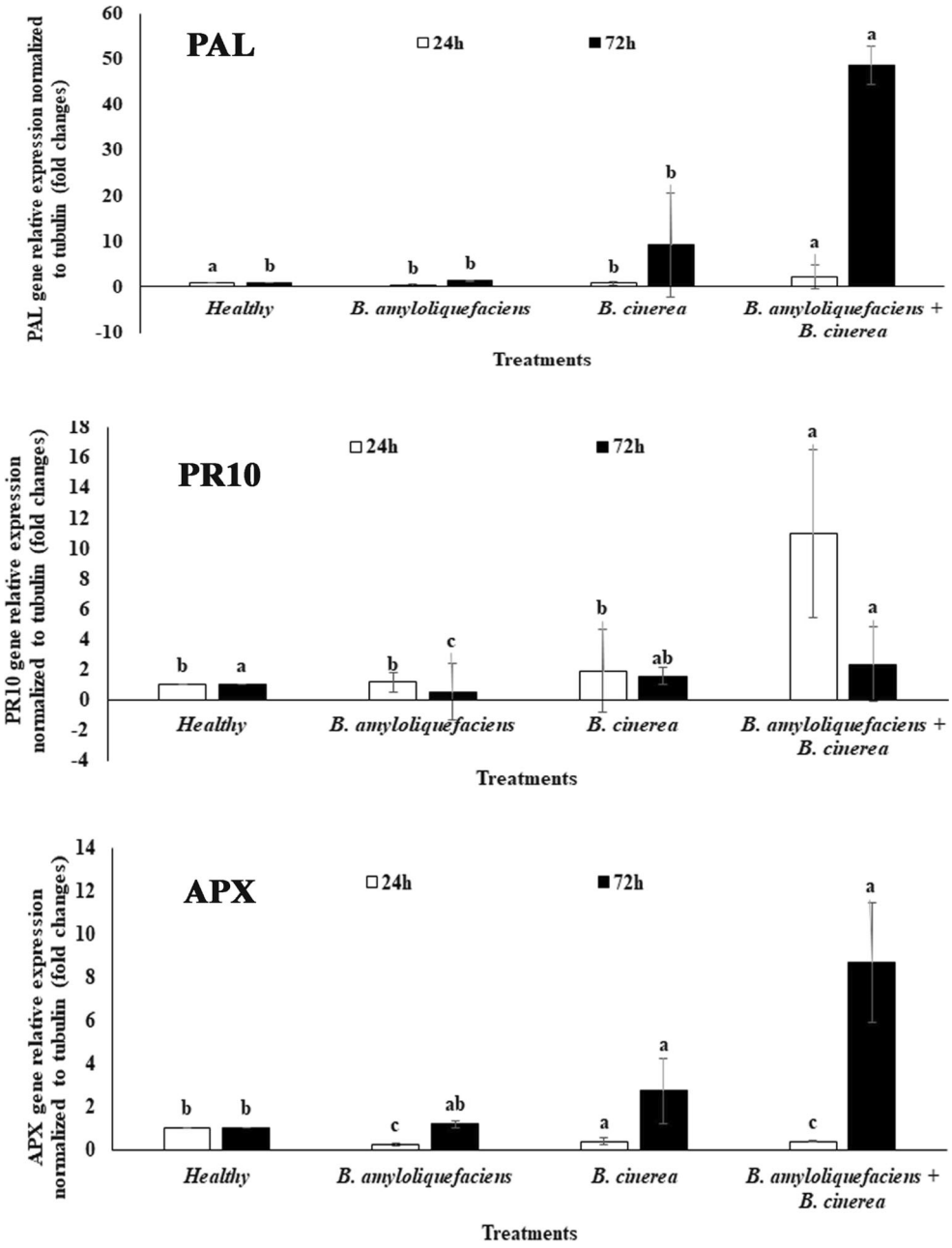

Fig. 2 Real-time quantitative PCR expression analysis of PAL, PR10 and ascorbate peroxidase genes at different day intervals after treatment with *Bacillus amyloliquefaciens* VB7, *Botrytis cinerea* strain (SEL) and the plants treated with *B. amyloliquefaciens* VB7 challenge inoculated *with B. cinerea* strain (SEL)

(2013) reported the effectiveness of *Bacillus* sp. BS061 against the mycelial growth of *B. cinerea*. Volatile metabolites of *B. subtilis* have a greater inhibitory effect on the mycelial growth of *Alternaria alternata, Paecilomyces lilacinus, Pythium afertile, P. variotii* and *Cladosporium oxysporum* than the diffusible NVOCs (Chaurasia et al. 2005). VOCs produced by *Pseudomonas chlororaphis* inhibited sclerotial germination and mycelial growth of *S. sclerotiorum* (Fernando et al. 2005). Lim et al. (2017) demonstrated the antifungal efficacy of VOCs produced by *Bacillus velezensis* against *B. cinerea*, *C. coccodes*, *F. oxysporum* f. sp. *lycopersici*, *M. oryzae*, *P. infestans*, *P. ultimum*, *R. solani* and *S. sclerotiorum*. Similarly, the non-volatile decenoic acid derivatives and pyrrolo chloroxylenol produced by *B. amyloliquefaciens* VB7 effectively reduced carnation stem rot incited by *S. sclerotiorum* under protected conditions (Vinodkumar et al. 2017). Besides, the antifungal volatile metabolites produced by *B. amyloliquefaciens* include dimethyl disulphide (Ossowicki et al. 2017), dimethyl trisulphide (Kai et al. 2009), tetradecane (Yuan et al. 2012) and hexadecane (Akpuaka et al. 2013).

In the present study, diversity in the expression of antifungal and antibacterial VOCs was observed in *B. amyloliquefaciens* VB7 cultured on the heat-killed mycelial biomass of *B. cinerea* (SEL) rather than the *B. amyloliquefaciens* VB7 cultured without the heat-killed mycelial biomass of *B. cinerea* (SEL). Similarly, the VOCs produced by *B. subtilis* have strong antifungal activity

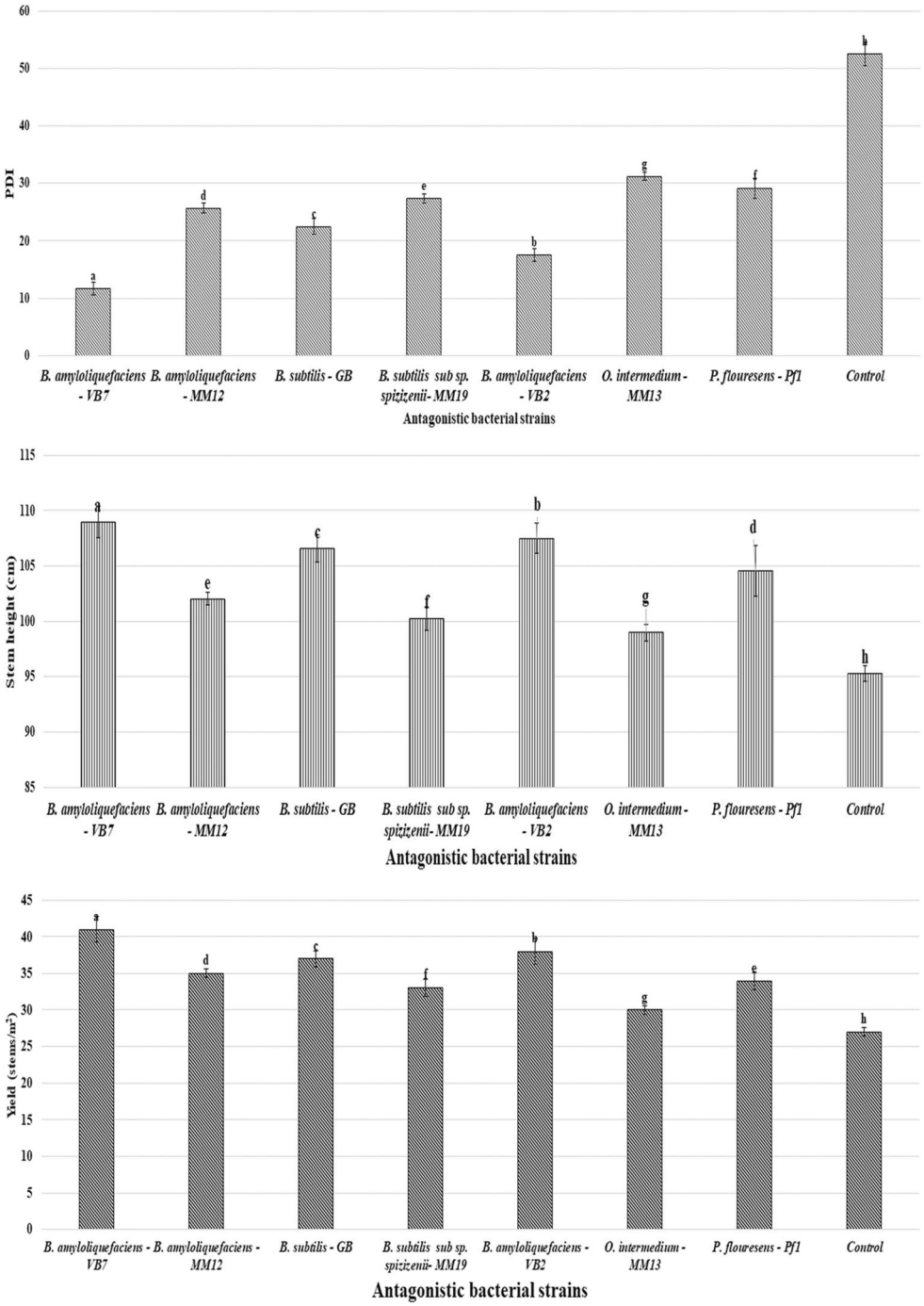

Fig. 3 Efficacy of bacterial strains on percentage leaf blight index, stem height and yield of *Lilium*. Data represent the mean value for two season trials (2015–2016 and 2016–2017). Analysis of variance was performed through DMRT with IRRISTAT (version.3/93, Biometrics Unit, International Rice Research Institute)

against fungal pathogens (Leelasuphakul et al. 2008). The VOCs 2,4-di-tertbutylphenol, 1-octanol and benzothiazole were effective in the suppression of *Colletotrichum gloeosporioides*, *Aspergillus niger* and *Penicillium chrysogenum* (Varsha et al. 2015). Similarly, Dheepa et al. (2016) demonstrated the antifungal nature of non-volatile compounds benzene, 1-dodecene, benzaldehyde, 1-dodecene, nonadecene, octadecanoic acid and n-tetradecene produced by *B. subtilis* (BS2) against *Puccinia horiana* in chrysanthemum. Apart from the volatile compounds produced by *B. amyloliquefaciens* VB7, it also produced antifungal non-volatile metabolites including phthalic acid, hept-3-yl isobutyl ester, phthalic acid and di(2-propylpentyl) ester. Similarly, several NVOCs from *Bacillus* sp. have been well demonstrated for their antimicrobial activity (Srinivasan et al. 2009; Klingler and Ebertz 2005; Khatiwora et al. 2012). However, *B. amyloliquefaciens* VB7 cultured on the heat-killed biomass of *B. cinerea* (SEL) produced more of antifungal compounds such as propanoic acid, 2-hydroxy-, methyl ester, phthalic acid, hept-4-yl isobutyl ester; phthalic acid, butyl 2-pentyl ester; phthalic acid, di(2-propylpentyl) ester and

hexadecanoic acid, methyl ester. 1,2-Benzenedicarboxylic acid and butyl 2-methylpropyl ester are antimicrobial in nature (Santhi et al. 2013), whereas hydroxymethylfurfural is antimicrobial only during fermentation (Yang et al. 2009). Benzothiazole released by *Bacillus velezensis* as a VOC inhibited the growth of *Monilinia fructicola.* Besides, it has the potential as a promising bioagent for the management of grey mould and tomato early blight (Gao et al. 2017). Likewise, *B. amyloliquefaciens* VB7 could also be explored for the management of *B. cinerea* causing *Lilium* leaf blight.

Bipartite and tripartite interactions of *B. amyloliquefaciens* VB7 and *B. cinerea* (SEL) in *Lilium* plants revealed the presence of antimicrobial and antifungal compounds such as 1,4-benzenedicarboxylic acid, bis(2-methylpropyl) ester (Rani et al. 2011) and phytyl palmitate, and diisooctyl phthalate apart from phthalic acid esters. However, the compounds bis(2-ethylhexyl) phthalate; thioxol-2-one, 4-(4-nitrophenyl)-; n-hexadecanoic acid; 9,12,15-octadecatrienoic acid, methyl ester, (Z, Z, Z)- and butyl 4,7,10,13,16,19-docosahexaenoate were detected during bipartite interaction. Similarly, Keskin et al. (2012) reported the production of antimicrobial compounds such as hexadecanoic acid, trisiloxane and octamethyl during the bipartite interaction of *B. cinerea* (SEL) with *Lilium* plants. However, the tripartite interaction of *Lilium* plants pre-treated with *B. amyloliquefaciens* VB7 followed by challenge inoculation of *B. cinerea* produced n-hexadecanoic acid, phthalic acid derivatives with antifungal nature, and ethyl 3-nitrobenzoate (Rehman et al. 2013) trisiloxane, octamethyl (Kim et al. 2015) with antibacterial nature. The effective suppression of foliar pathogens requires survival and colonization of the biocontrol agent on plant surfaces (Ramey et al. 2004). Colonization by the bacterial antagonists on the phylloplane needs biofilm formation, which is an adaptive response to counter the establishment of the host–pathogen relationship in the phylloplane. Phylloplane colonization and the production of lipopeptides by the antagonistic *B. subtilis* were effective in the suppression of *Podosphaera fusca* causing cucurbit powdery mildew (Romero et al. 2004; Zeriouh et al. 2011). Biofilm formation mediated through surfactin colonized leaf surface of melon and suppressed powdery mildew (Zeriouh et al. 2013). Similarly, *B. amyloliquefaciens* VB7 formed biofilms on the phylloplane, colonized the conidia of *B. cinerea* and thus prevented the germination of the *Botrytis* leaf blight pathogen of *Lilium*. Exposure of *B. cinerea* to the culture filtrate of *B. cereus* strain B-02 induced complete distortion and shrinkage of conidia along with swelling of hyphae (Li et al. 2012). This indicates that the interaction of *B. amyloliquefaciens* VB7 with the conidia of *B. cinerea* might be responsible for the shrinkage of conidia, via the production of antimicrobial peptides in the phylloplane.

Studies on defence-related gene expression revealed increased transcript levels of PAL, PR10 and APX against *B. cinerea* challenged with *B. amyloliquefaciens* VB7. Several earlier studies also correlated the induction of defence-related genes by different bioagents with reduced disease severity and increased disease resistance in apple, Chinese bayberry, loquat and peach fruit (Wang et al. 2011; Cao et al. 2008; Chan and Tian 2005). Chandrasekaran and Chun (2016) reported the induction of the PAL gene by *B. subtilis* responsible for resistance to soft rot disease. Likewise, higher accumulation of PR proteins was observed in tomato challenge inoculated with *Methylobacterium* against *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* (Yim et al. 2014). Chen et al. (2006) reported that maize crop was protected against *A. flavus* by inducing the expression of the zmPR-10 gene. This is in line with PR-10 expression in rice against *M. grisea* (McGee et al. 2001) and cowpea against the infection of *Uromyces vignae* (Mould et al. 2003). Abiotic stress induced the expression of *APX*-encoding genes (Bonifacio et al. 2011). From our investigation, we inferred that bulb dipping, soil application and foliar application of *B. amyloliquefaciens* VB7 (1% FS at 10^8 CFU/ml) significantly reduced disease incidence and increased plant growth parameters. This result is in accordance with the findings of Vinodkumar et al. (2017) who reported that strain VB7 was effective in the management of *S. sclerotiorum* both in vitro and in protected conditions. They also detected 10 different AMP genes in *B. amyloliquefaciens* strain VB7. Chiou and Wu (2003) also reported that the strains of *B. amyloliquefaciens* effectively controlled grey mould of lily in field conditions.

This study confirmed the effectiveness of *B. amyloliquefaciens* VB7 through bulb dipping, soil application and as foliar spray at 10 ml/L (10^8 CFU/ml) with diverse mode of action for the management of *Botrytis* leaf blight of *Lilium* under protected conditions and revealed the rapid colonization of the bacterial cells of VB7 on the phylloplane coupled with the lysis of the conidia and induction of defence genes. Besides, the VOCs/NVOCs produced during bipartite and tripartite interactions may aid in the suppression of *Botrytis* leaf blight. In conclusion, *B. amyloliquefaciens* VB7 can be used as an effective antagonist for growth promotion and *Botrytis* leaf blight management.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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