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Antifungal effects of compost tea microorganisms on tomato pathogens

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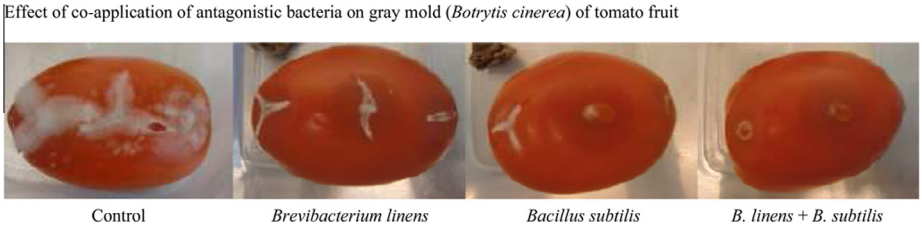
HIGHLIGHTS

- Bacteria from compost tea reduced mycelial growth of tomato pathogens.
- *Bacillus subtilis* and *Brevibacterium linens* inhibited disease on tomato fruit.
- Combined bacterial application revealed synergistic effects.
- *Bacillus subtilis* produced antifungals from the surfactin family of lipopeptides.

GRAPHICAL ABSTRACT



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ABSTRACT

Compost teas are fermented aqueous extracts of composted materials that are used for their ability to control plant pathogens. It had been previously reported that this inhibition by compost teas is at least partially attributed to the presence of live microorganisms. In this study, the inhibitory effects of bacteria from suppressive compost tea were examined against mycelial growth of *Alternaria solani* and *Botrytis cinerea* as well as disease development on tomato fruit. Isolation of antifungal extracts and identification of antifungal compounds from the most effective bacterial strains were also performed. Results showed that the bacteria had the ability to greatly inhibit the mycelial growth of *B. cinerea* and/or *A. solani* by up to 70%. The two most effective isolates, *Brevibacterium linens* (IC 10) and *Bacillus subtilis*, showed that co-application of bacterial antagonists (5×10^5 or 5×10^6 cells) with the pathogens on tomato fruit demonstrated inhibition of the development of *B. cinerea* lesions by up to 61%. A preventive application of the bacteria (5×10^5 or 5×10^6 cells) was more effective than co-application, allowing a significant reduction in lesions of *A. solani* and improving efficacy of low bacterial concentrations in reducing *B. cinerea* lesions. A combined *B. linens*/*B. subtilis* treatment was generally more inhibitory than either bacterium alone indicating possible synergistic effects. Antifungal compounds, including surfactins, were found in the bacterial extracts indicating that antibiosis is a main mechanism of action.

1. Introduction

Synthetic fungicides remain the most widely used control measure against fungal plant diseases. Although relatively effective, synthetic fungicides have two major drawbacks: their generally widespread lack of long-term efficacy caused by the development of resistance in plant pathogens (Avis, 2007) and their potential adverse effects on human health and the environment (Kolaei et al., 2013). In addition to these drawbacks in field or greenhouse cropping systems, pesticide treatments in warehouses and cold storage units have the added constraints of few newly registered synthetic chemicals as well as few replacements for some banned chemicals (Janisiewicz and Korsten, 2002). Therefore, there remains an urgent need for efficient and reliable pre- and post-harvest plant disease control measures.

A possible alternative to synthetic chemical fungicides is to exploit the antimicrobial activities of compost teas. Compost teas, which are considered safer for health and the environment

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(Siddiqui et al., 2009), are fermented watery extracts of composted materials that can be used for the control of plant pathogens (Litterick et al., 2004). Recently, compost teas from sheep manure compost showed antimicrobial activities against phyllosphere (Koné et al., 2010) and rhizosphere (Dionne et al., 2012) pathogens of tomato (*Solanum lycopersicum* L.) plants. These composts teas did not contain human pathogens (Koné et al., 2010). This and other work indicated that the microbial populations within compost teas were the main factor responsible for the inhibitory effects of compost teas (Scheuerell and Mahaffee, 2004, 2006; Diánez et al., 2006; Gea et al., 2009; Koné et al., 2010; Dionne et al., 2012).

Beneficial microbial antagonists in suppressive compost teas are reported to control plant pathogens through one or more biological control mechanisms. Indeed, the microorganisms present in the tea may act as pathogen antagonists through their ability to compete for nutrients and/or space (Al-Mughrabi et al., 2008), to destroy pathogens by parasitism (El-Masry et al., 2002), to induce systemic resistance in plants (Zhang et al., 1998), and/or to produce antimicrobial compounds (*i.e.*, antibiosis). Microbial antagonists presumably using antibiosis as a mechanism of biocontrol were isolated from sheep manure compost tea (Koné et al., 2010; Dionne et al., 2012) by targeting their ability to produce extracellular antimicrobial compounds.

In this study, the antagonistic bacteria isolated from sheep manure compost tea were tested (i) to assess their inhibitory activity on mycelial growth of fungal pathogens, (ii) to evaluate their ability to suppress disease *in vivo*, and (iii) to isolate and identify antifungal compounds from the most effective bacterial strains. This study was conducted with the plant pathogenic fungi *Alternaria solani* Sorauer and *Botrytis cinerea* Pers.:Fr. *A. solani* causes disease of potato (*Solanum tuberosum* L.), eggplant (*Solanum melongena* L.), pepper (*Capsicum annuum* L.), and tomato (*S. lycopersicum* L.) and is generally referred to as early blight disease (Zhao et al. 2008). *B. cinerea* is the causal agent of gray mold on numerous plant species including tomato (Cantu et al., 2009).

2. Materials and methods

2.1. Microbial material

The antagonistic bacteria were isolated from a compost tea prepared from sheep manure compost (Koné et al., 2010; Dionne et al., 2012). *Advenella incenata* Coenye et al. isolates IB48 (GenBank Accession number KJ689306) and IC6 (KJ689309), *Aminobacter aminovorans* (den Dooren de Jong) Urakami et al. isolate IB2 (KJ689311), *Bacillus subtilis* (Ehrenberg) Cohn isolate IC23 (KJ689307), and *Brevibacterium linens* (Wolff) Breed isolates IB16 (KJ689310) and IC10 (KJ689308) were identified using nucleotide sequence data from the small subunit (16S) of ribosomal RNA. These bacteria were cultured from freeze-dried or glycerol stocks and maintained on tryptic soy agar (TSA, Becton Dickinson, Sparks, MD). The fungal plant pathogens *A. solani* and *B. cinerea* were provided by the Laboratoire de diagnostic en phytoprotection (MAPAQ, Québec, Canada) and were cultured from freeze-dried stocks and maintained on potato dextrose agar (PDA, Becton Dickinson).

2.2. Effect of compost tea antagonists on the mycelial growth of *B. cinerea* and *A. solani*

Bacteria were transferred from TSA Petri dishes to 15-mL conical tubes containing 5 mL of tryptic soy broth (TSB, Becton Dickinson) using an inoculation loop. The bacteria were grown in an incubator-shaker for 48 h at 26 °C and 175 rpm. Following incubation, bacteria were adjusted to 5×10^7 cells/mL with sterile distilled water using a hemacytometer. Ten microliter of each bacterial suspension was inoculated as a single streak (1 cm wide) at the four cardinal points of a Petri dish containing PDA. The distance between two opposite streaks was 6.5 cm (for *B. cinerea* trials) and 4.5 cm (for *A. solani* trials). The dishes were incubated for 48 h at 26 °C in the dark. Following this incubation period, the center of each dish was inoculated with a 0.5-cm (diameter) agar plug containing actively growing mycelia from the thallus margins of either *B. cinerea* or *A. solani*. PDA inoculated with either *B. cinerea* or *A. solani*, but without the bacteria served as controls. The dishes were incubated another 48 h at 26 °C. The mycelial growth of *B. cinerea* and *A. solani* was noted after the incubation period and expressed as the average of two perpendicular diameters of the thallus. The experiments were conducted as complete block designs with five replicates.

2.3. Effect of *B. linens* (IC10) and *B. subtilis* on disease on tomato fruit

2.3.1. Effect of co-application of *B. linens* (IC10) and *B. subtilis* on disease of tomato fruit

B. linens (IC10) and *B. subtilis* were transferred from TSA Petri dishes to 15-mL conical tubes containing 5 mL of tryptic soy broth (TSB, Becton Dickinson) using an inoculation loop. The bacteria were grown in an incubator-shaker for 48 h at 26 °C and 175 rpm. Following incubation, bacteria were recovered by centrifugation (5300×*g* for 20 min). The supernatant was removed and the pellet was washed with 5 mL of sterile distilled water and recentrifuged. The supernatant was discarded and the remaining pellet served as inoculum stocks. The bacterial inocula were adjusted to 5×10^7 cells/mL or 5×10^8 cells/mL with sterile distilled water using a hemacytometer.

A. solani and *B. cinerea* spores were individually collected by flooding mycelia grown for 1 (*A. solani*) and 2 weeks (*B. cinerea*) on PDA with 1 mL sterile distilled water and by gently scraping the surface of the mycelia with a sterile glass rod to dislodge the spores. The spore suspensions were collected by aspiration with a micropipette, placed in a 1.5-mL microcentrifuge tube and diluted to 5×10^4 conidia/mL based on hemacytometer counts of the stock suspension.

Tomato fruit (cultivar 'Roma') were surface sanitized in 70% ethanol for 15 min, rinsed with sterile distilled water, and allowed to air dry. The skin of each tomato was pierced with a sterile needle to provide a wounded inoculation site (diameter 1.5 mm, depth 7 mm). Each wound was inoculated individually with 10 μL of fungal spore suspension (*A. solani* or *B. cinerea*). Following fungal inoculation, wounds were immediately treated with either 10 μL of sterile distilled water (controls), 10 μL of *B. linens* IC10 cell suspension, 10 μL of *B. subtilis* cell suspension or 5 μL each of *B. linens* IC10 and *B. subtilis*. Suspensions were used at the concentration indicated above to provide 1000:1 and 10,000:1 bacterial cell/fungal spore ratios as previously described (Siripornvisal, 2010). In addition, 10 μL of each bacterial cell suspension was tested in the absence of fungal spores to assess the potential effect of the bacteria on the tomato fruit. Each tomato fruit was individually placed in a plastic container with a moistened paper towel to maintain relative humidity (RH > 95%). Containers were sealed and incubated in the dark at 20 °C for five (*B. cinerea*) to seven (*A. solani*) days. Symptoms of disease were measured as the average of two perpendicular diameters of the visible surface lesions following 5 and 7 days of incubation for *A. solani* and 3 and 5 days of incubation for *B. cinerea*. The experiments consisted of randomized complete block designs with three wounds per tomato and three replicates per treatment. The experimental unit was one tomato fruit in an individual container. The experiments were repeated twice.

2.3.2. Effect of preventive application of B. linens IC10 and B. subtilis on disease of tomato fruit

In this trial, bacteria were allowed to establish prior to inoculation with the fungal pathogens in order to evaluate the effect of preventive bacterial treatments on *A. solani* and *B. cinerea* as follows. Tomato fruit were surface sterilized and wounded as described previously. Wounds were inoculated with either 10 μL of sterile distilled water (controls), 10 μL of *B. linens* IC10 cell suspension, 10 μL of *B. subtilis* cell suspension or 5 μL each of *B. linens* IC10 and *B. subtilis*. Following bacterial inoculations, tomato fruit were incubated in plastic containers at 20 °C (RH > 95%) in the dark for 24 h. Subsequently, 10 μL of *B. cinerea* or *A. solani* spore suspensions were inoculated into the wounds. Suspensions were used to provide a 1000:1 and 10,000:1 bacterial cell/fungal spore ratios as described above. The tomatoes were incubated in the same conditions for 5 (*B. cinerea*) or 7 (*A. solani*) days. Lesion sizes were measured as described previously on days 5 and 7 for *A. solani* and on days 3 and 5 for *B. cinerea*. The experiments consisted of randomized complete block designs with three wounds per tomato and three replicates per treatment. The experimental unit was one tomato fruit in an individual container. The experiments were repeated twice.

2.4. Antifungal compounds from B. linens IC10 and B. subtilis

2.4.1. Extraction of potential antifungal compounds

TSA dishes were streaked with *B. linens* IC10 or *B. subtilis* on one half of each dish. The dishes were incubated for 24 h at 26 °C. Agar plugs containing actively growing mycelium of *B. cinerea* were placed in the center of the remaining half of each dish. The dual confrontation culture dishes were incubated for an additional 48 h at 26 °C. Following the confrontation period, the TSA containing the bacteria and the visible inhibition zone was recovered, cut into cubes, lyophilized, and ground to a powder.

Dried and ground samples were placed into 15-mL conical tubes and solid-liquid extraction was performed with 80% methanol at a concentration of 10 mL/g of sample. The samples were extracted for 24 h at 20 °C and with shaking (150 rpm). The samples were vacuum-filtered on filter paper (Whatman paper #42; 2.5 μm pore size; 9 cm diameter) and/or centrifuged at 5,300 × *g* for 20 min to remove the bacteria and any other insoluble material in order to recover the filtrates/supernatant. The filtrates/supernatants were then filtered on 0.45-μm filter (flask or syringe) to obtain a cell-less extract. The extracts were evaporated to dryness on a rotary evaporator and/or under a flow of nitrogen. The extracts were resuspended in 80% methanol to 50 × their initial concentration.

In addition to extraction of antifungal compounds from dual confrontation cultures, *B. subtilis* was also cultured on medium for optimal lipopeptide production (MOLP). The medium was precipitated with ammonium sulfate [$(NH_4)_2SO_4$] to recover antimicrobial lipopeptides as described by Akpa et al. (2001).

Extracts from MOLP cultures were separated by reverse-phase chromatography using a 1 g (5 mL) Bond Elut C_{18} SPE cartridge (Varian, Palo Alto, CA). The cartridge was conditioned with two volumes (10 mL) of ultrapure water prior to the application of the concentrated extracts. Compounds were sequentially eluted with 1 volume (5 mL) of increasing concentrations of methanol:water as follows: 0:100, 20:80, 50:50, 80:20, 100:0 (v/v). Fractions were individually collected and tested for antifungal activity as described below.

2.4.2. Antifungal assays

2.4.2.1. Disk diffusion assays. For *B. linens* IC10 and *B. subtilis* methanol extracts, 50 μL of each crude extract was placed on sterile paper disks (Whatman paper #1; 0.7 cm diameter). A combined *B. linens*/*B. subtilis* extract treatment was used containing 25 μL of each crude extract on the same disk. 80% methanol served as the control. Following drying of the extracts, disks were placed on a PDA Petri dish. Plugs covered with *B. cinerea* or *A. solani* were then placed 4 (*B. cinerea*) or 2.5 cm (*A. solani*) from the paper disks. The plates were incubated for 72 h at 20 °C and the zone of inhibition on each plate was measured. The zone of inhibition was measured as the distance between the paper disk and edge of the fungal growth. The experiments consisted of randomized complete block designs with three replicates per treatment.

2.4.2.2. Spore germination assays. B. linens IC10 and *B. subtilis* crude methanol extracts and the MOLP fractions were further tested for their effect on fungal spore germination (inhibition of germ tube emergence and/or development). Each crude extract or fraction was dried on a roto-evaporator and/or under a stream of nitrogen and dissolved in 1 mL sterile distilled water. Spore suspensions of *A. solani* and *B. cinerea* were prepared separately in sterile distilled water and serial diluted to 5×10^3 spores/mL. In a sterile 2-mL microcentrifuge tube, 80 μL of 2× concentrated potato dextrose broth (Becton Dickinson) and 20 μL of spore suspension were combined with 100 μL crude extract, MOLP fraction or sterile distilled water (control). The tubes were incubated at 20 °C in the dark for 3 days. Germination was appraised by visual assessment of macroscopic growth in the tube on a 0–4 scale were 0 = no germination/growth and 4 = complete germination/growth. The experiments consisted of randomized complete block designs with three replicates.

2.4.3. Identification of antifungal compounds

Bioactive fractions from *B. subtilis* cultured in MOLP were subjected to analysis of their potential antifungal lipopeptides by LC–ESI-MS analysis. Approximately 200 μL of each antifungal fraction was filtered through 0.45-μm syringe filters and transferred to LC vials.

LC–ESI-MS was performed using a Waters 2795 separations module and MicroMass Quatro Ultima triple quadrupole mass spectrometer. Fractions were separated by a Phenomenex Kinetix C_{18} (100 × 4.6 mm, 2.6 μm, 100 Å) column (Torrance, CA) using a mobile phase consisting of acetonitrile–water (ACN–H_2O) containing formic acid [0.1%, (v/v)]. The linear solvent gradient was from 5% to 100% ACN over 13 min with a flow rate of 1 mL min^{-1}. Positive ESI conditions included: capillary voltage 3.50 kV, cone voltage 20 V, source temperature 80 °C, desolvation temperature 180 °C, cone gas flow (N_2) 90 L/h, desolvation gas flow (N_2) 540 L/h, and multiplier voltage of 650 V. The mass range examined was *m*/*z* 900–1600. Retention times and MS data of the extracts were compared to authentic *B. subtilis* lipopeptide standards fengycin A (AIBI, AUG, Belgium), iturin A (Sigma–Aldrich, Mississauga, Ontario, Canada), and surfactin (Sigma–Aldrich).

2.5. Statistical analysis

For the *in vitro* trial in Petri dishes, the *in vivo* assays on tomato fruit, the disk diffusion and spore germination assays, analysis of variance (ANOVA) was performed using the GLM procedure in SAS (SAS Institute, 2004). When significant ($P \leqslant 0.05$), means were separated using Fisher's protected least significant difference (LSD) test at α level = 0.05. For the repeated experiments (i.e., the *in vivo* trials on tomato fruit), ANOVA indicated that there was no significant difference between the two experiments. These data were therefore combined and analyzed as a single experiment.

3. Results

3.1. Effect of compost tea antagonists on the mycelial growth of *B. cinerea* and *A. solani*

Compost tea bacteria generally inhibited the growth of both fungi (Table 1). In *B. cinerea* trials, *B. linens* (IC10) and *B. subtilis* revealed the highest inhibitory activity, decreasing mycelial growth by 70%. *A. incenata* (IC6) and *B. linens* (IC16) reduced *B. cinerea* growth by approximately 27%. Finally, *A. incenata* (IB48) and *A. aminovorans* also significantly inhibited *B. cinerea* growth, albeit by only 15%.

In *A. solani* trials, *B. linens* (IC10) and *B. subtilis* showed the highest inhibitory effect reducing mycelial growth of the fungus by approximately 57%. *A. incenata* (IC6), *B. linens* (IB16), and *A. aminovorans* reduced *A. solani* growth by approximately 35%, 26%, and 10%, respectively. *A. incenata* (IB48) did not inhibit the mycelial growth of *A. solani* (Table 1).

3.2. Effect of co-application of *B. linens* (IC10) and *B. subtilis* on disease of tomato fruit

In absence of pathogen inoculation, application of the bacteria alone did not reveal spoilage (lesions, unsightly bacterial growth or offensive odors) on the tomato fruit over a 7-day incubation period.

With fungal pathogen inoculations, results showed that *B. linens* (IC10) and *B. subtilis* had variable effects on fungal disease. No bacterial treatments showed significant inhibition ($P > 0.05$) of *A. solani* lesions over a 7-day period at either concentration ratio (Fig. 1).

At a 1000:1 bacterial cell/fungal spore ratio, the individual bacterial treatments did not reduce lesions of *B. cinerea* after 3 days of incubation. Conversely, the combined *B. linens*/*B. subtilis* treatment significantly reduced *B. cinerea* lesions by 58% when compared to the control after 3 days (Fig. 2). At the same ratio, all treatments significantly reduced *B. cinerea* lesions after 5 days of incubation. Indeed, individual treatment of *B. linens* or *B. subtilis* inhibited *B. cinerea* lesions by approximately 20%. The combined *B. linens*/*B. subtilis* treatment provided the highest inhibition (44%) after a 5-day incubation period (Fig. 2). When the ratio was increased to 10,000:1, all treatments significantly reduced *B. cinerea* lesions. Indeed, all three bacterial treatments reduced lesions of gray mold by 42% after 3 days (Fig. 2). After a 5-day incubation, the individual treatment of *B. linens* or *B. subtilis* inhibited *B. cinerea* lesions by 41% whereas the combined *B. linens*/*B. subtilis* treatment provided the highest inhibition (61%) of the disease (Fig. 2).

3.3. Effect of preventive application of *B. linens* IC10 and *B. subtilis* on disease of tomato fruit

At a bacterial cells/fungal spores ratio of 1000:1, bacterial treatments did not inhibit *A. solani* lesions over a 7-day period (Fig. 3).

Table 1
Effect of bacterial antagonists on the mycelial growth of fungal pathogens.

	Mycelial growth (cm)			
	Botrytis cinerea		*Alternaria solani*	
Control	6.17	a	4.08	a
Advenella incenata (IB48)	5.20	b	3.79	ab
Advenella incenata (IC6)	4.49	c	2.65	d
Aminobacter aminovorans (IB2)	5.27	b	3.68	b
Bacillus subtilis (IC23)	1.86	d	1.87	e
Brevibacterium linens (IB16)	4.50	c	3.02	c
Brevibacterium linens (IC10)	1.93	d	1.65	e

Values are diameter of the thallus.
Within a column, means followed by the same letter are not significantly different according to Fisher's protected LSD test (α level = 0.05).

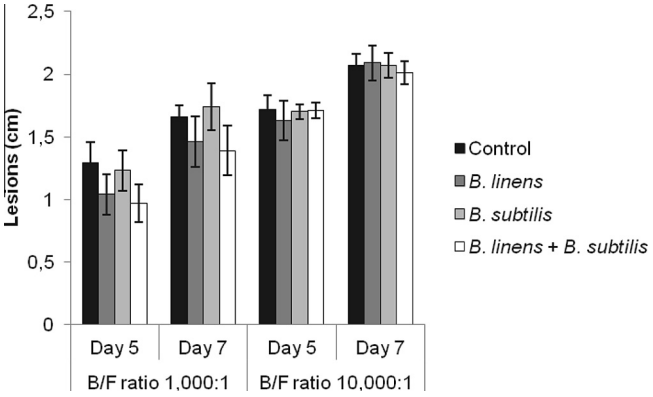

Fig. 1. Effect of co-application of *Brevibacterium linens* (IC10) and/or *Bacillus subtilis* on lesions (diameter) caused by *Alternaria solani* on tomato fruit. Days are incubation period following inoculations. B/F ratios are bacterial cell/fungal spore inoculum ratios. Error bars are SE. Within each grouping, values are not significantly different ($P > 0.05$).

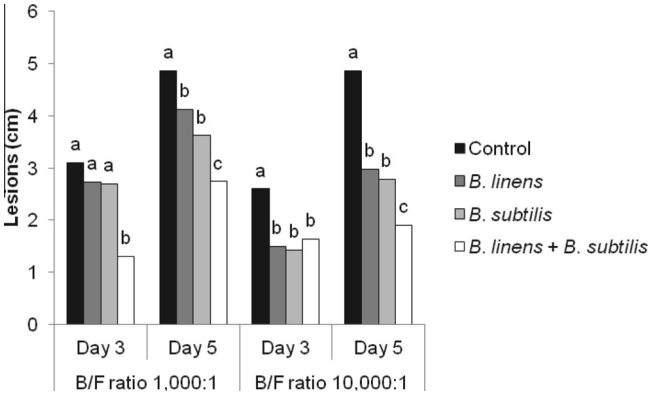

Fig. 2. Effect of co-application of *Brevibacterium linens* (IC10) and/or *Bacillus subtilis* on lesion (diameter) caused by *Botrytis cinerea* on tomato fruit. Days are incubation period following inoculations. B/F ratios are bacterial cell/fungal spore inoculum ratios. Within each grouping, values with a letter in common are not significantly different according to Fisher's protected LSD test (α level = 0.05).

At the higher bacterial cell/fungal spore ratio (10,000:1), the combined *B. linens*/*B. subtilis* treatment significantly inhibited *A. solani* lesions by 22% after 5 days of incubation. Treatment with either bacterium alone did not provide significant inhibition at this incubation period. Following a 7-day incubation at this same bacteria cells/fungal spores ratio, treatment with *B. subtilis* significantly inhibited *A. solani* lesions by 16% whereas the combined *B. linens*/*B. subtilis* treatment showed the highest efficacy, reducing *A. solani* lesions by 36% (Fig. 3).

At a 1000:1 bacterial cell/fungal spore ratio, all bacterial treatments almost equally reduced lesions of *B. cinerea* by approximately 63% and 44%, after 3 and 5 days of incubation, respectively (Fig. 4). When the bacterial ratio was increased to 10,000:1, all treatments significantly reduced *B. cinerea* lesions when compared to the control by 56%, 42%, and 64% for *B. linens*, *B. subtilis*, and the combined *B. linens*/*B. subtilis* treatments, respectively, after a 3-day incubation. After a 5-day incubation, the *B. subtilis* treatment significantly reduced *B. cinerea* lesions by 42% whereas the *B. linens* treatment and the combined *B. linens*/*B. subtilis* treatment provided the highest inhibition of the fungus (56% and 64%, respectively) (Fig. 4).

3.4. Antifungal extracts from *B. linens* IC10 and *B. subtilis*

In order to determine the production of antifungal compounds by *B. linens* (IC10) and *B. subtilis*, methanolic extracts were assessed

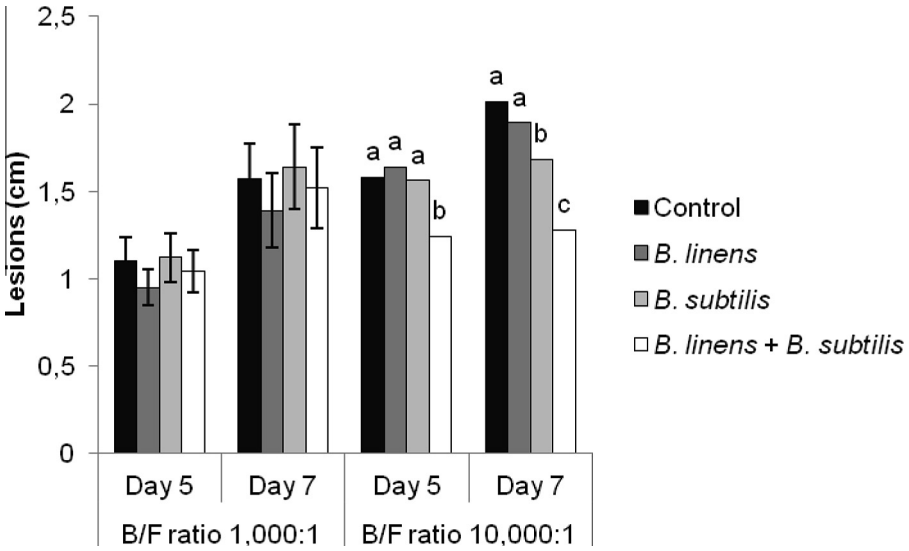

Fig. 3. Effect of a preventive application of *Brevibacterium linens* (IC10) and/or *Bacillus subtilis* on lesions (diameter) caused by *Alternaria solani* on tomato fruit. Days are incubation period following inoculations with *A. solani*. B/F ratios are bacterial cell/fungal spore inoculum ratios. Error bars are SE. Within each grouping with error bars, values are not significantly different ($P > 0.05$). Within each grouping with letters, values with a letter in common are not significantly different according to Fisher's protected LSD test (α level = 0.05).

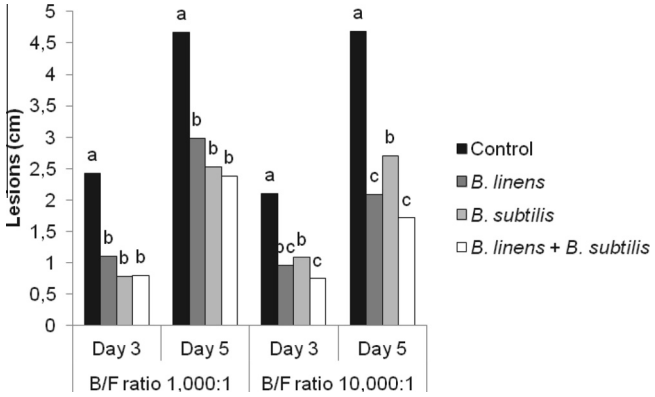

Fig. 4. Effect of a preventive application of *Brevibacterium linens* (IC10) and/or *Bacillus subtilis* on lesions (diameter) caused by *Botrytis cinerea* on tomato fruit. Days are incubation period following inoculations with *B. cinerea*. B/F ratios are bacterial cell/fungal spore inoculum ratios. Within each grouping, values with a letter in common are not significantly different according to Fisher's protected LSD test (α level = 0.05).

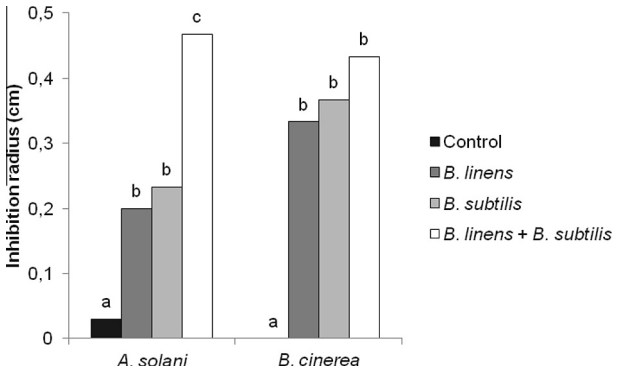

Fig. 5. Effect of extracts from *Brevibacterium linens* (IC10) and/or *Bacillus subtilis* on radial mycelial growth of *Alternaria solani* and *Botrytis cinerea* after 3 days incubation at 20 °C in the dark. Within each grouping, values with a letter in common are not significantly different according to Fisher's protected LSD test (α level = 0.05).

for inhibition of mycelial growth and spore germination of *B. cinerea* and *A. solani*. In disk diffusion assays, individual bacterial extracts showed significant inhibition zones against the mycelia of both fungi (Fig. 5). The combined *B. linens*/*B. subtilis* treatment showed inhibition equivalent to that of the individual extracts in *B. cinerea* whereas it showed the highest inhibitory effect against *A. solani* (Fig. 5).

Similar to the diffusion assays, spore germination assays showed that individual bacterial extracts significantly inhibited both fungi (Fig. 6). Moreover, the combined *B. linens*/*B. subtilis* treatment showed the highest inhibitory effect to spores of both *A. solani* and *B. cinerea* (Fig. 6).

3.5. Antifungal compounds from B. subtilis

The main antifungal activity was found in the fraction eluted with the 100% methanol. This fraction was subsequently analyzed by LC–ESI-MS. The total ion count chromatogram (TIC) ion positive mode revealed the presence of two major peaks at 13.51 and

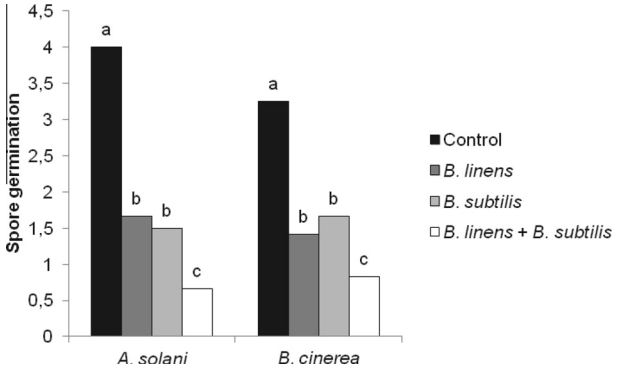

Fig. 6. Effect of extracts from *Brevibacterium linens* (IC10) and/or *Bacillus subtilis* on spore germination of *Alternaria solani* and *Botrytis cinerea* after 3 days incubation at 20 °C. Germination was appraised on a 0–4 scale were 0 = no germination/growth and 4 = complete germination/growth. Within each grouping, values with a letter in common are not significantly different according to Fisher's protected LSD test (α level = 0.05).

13.93 min. MS data of the first peak (13.51 min) showed two pseudo-molecular ion peaks at *m*/*z* 1022 [M+H]+ and 1044 [M+Na]+, which were attributed to the C_{14} homolog of surfactin. The second peak (13.93 min) from TIC chromatogram displayed both protonated and sodiated ion peaks in the MS spectrum at *m*/*z* 1036 [M+H]+ and 1058 [M+Na]+, which were attributed to the C_{15} homolog of surfactin. Attribution of identity was based on comparison with the authentic standard as well as the literature (Pecci et al., 2010). LC–ESI-MS data did not show any evidence of the fengycin or iturin family of antimicrobial compounds.

4. Discussion

It has been previously found that plant pathogen inhibition by compost teas are at least partially attributed to the presence of live microorganisms (Scheuerell and Mahaffee, 2004, 2006; Diánez et al., 2006; Gea et al., 2009; Koné et al., 2010; Dionne et al., 2012). In this study, the antifungal effects of bacteria from sheep manure compost tea were examined against mycelial growth of *A. solani* and *B. cinerea* as well as disease lesions on tomato fruit. This study showed that the bacteria had the ability to greatly reduce the mycelial growth of *B. cinerea* and/or *A. solani*. Among the bacteria tested, *B. linens* (IC10) and *B. subtilis* gave the highest inhibition of both tested pathogens, reducing the mycelial growth from 57% to 70% (Table 1). Two *A. incenata* isolates, *A. aminovorans*, and another *B. linens* isolate (IB16) were also inhibitory to at least one of these molds, albeit to a lesser extent. Previous reports have shown that these antagonists have the ability to inhibit the mycelial growth of various plant pathogens. Indeed, *A. incenata* had been shown to inhibit the mycelial growth of *Phytophthora parasitica* Dastur var. *nicotianae* (Breda de Haan) Tucker (Jin et al., 2011). *B. subtilis* has been shown to affect the growth of numerous plant pathogens including *Peronophythora litchi* Chen ex Ko et al. (Jiang et al., 2001), *Gaeumannomyces graminis* var. *tritici* Walker, *Coniella diplodiella* (Speg.) Petr. & Syd., *Phomopsis* sp., and *Sclerotinia sclerotiorum* (Lib.) de Bary (Liu et al., 2009), *A. solani*, *B. cinerea*, *Fusarium sambucinum* Fuckel, and *Pythium sulcatum* R.G. Pratt & J.E. Mitch. (Wise et al. 2012). *B. linens* had previously shown mycelial inhibition of *Phytophthora vignae* Purss (Fernando and Linderman, 1994). To the best of our knowledge, this is the first report of *A. aminovorans* demonstrating antagonistic effects on mycelial growth of plant pathogens.

The two most effective isolates *in vitro*, *B. linens* (IC 10) and *B. subtilis*, were further tested for their inhibitory effects *in vivo* on disease of tomato fruit artificially-inoculated with *B. cinerea* or *A. solani*. Co-application of the bacteria failed to control *A. solani*. Conversely, co-application of bacteria frequently demonstrated inhibition of the development of *B. cinerea* lesions by up to 61% (Fig. 2). *B. subtilis* has been frequently shown to alleviate plant disease *in vivo* (Ongena and Jacques, 2008; Falardeau et al., 2013). Although there have been few reports, ability of *B. linens* to control disease in plant pathogens has been previously shown (Fernando and Linderman, 1993).

The preventive application of the bacteria improved the inhibitory effect of the bacteria on *A. solani* lesions. Indeed, the higher bacterial cell/fungal spore ratio showed that treatments with *B. subtilis* as well as the combined *B. linens*/*B. subtilis* treatment became inhibitory to *A. solani* when the bacteria were allowed to establish prior to pathogen inoculation. This may indicate that suppressive effects of bacteria are partly because of inhibition of fungal spore germination and/or penetration inside the plant cells as have been shown previously (Diánez et al. 2007; Siddiqui et al. 2009). At the lower bacterial cell/fungal spore ratio, a preventive application also generally improved the ability of the bacteria to control *B. cinerea* lesions. At the higher bacterial cell/fungal spore ratio, there was little difference in control between a co-application (Fig. 2) and a preventive treatment (Fig. 4) for *B. cinerea*. These results indicate that fungal disease pressure and bacterial concentration, as well as timing of the bacterial applications are important factors in the disease control outcome.

Of particular note, the combined *B. linens*/*B. subtilis* treatment was frequently more inhibitory than the same concentration of either bacterium alone. This would indicate the possible synergistic effects of both bacteria on improving control of a fungal pathogen as has been shown previously (Avis et al., 2008).

Extracts from dual confrontation cultures showed inhibitory effects against both mycelial growth and spore germination of *A. solani* and *B. cinerea* (Figs. 5 and 6). These results indicated that the bacteria produced extracellular antifungal compounds and that antibiosis may therefore be part of their mechanisms of action against fungi (Fravel, 1988). *B. subtilis* strains have been shown to produce numerous peptide (Ongena and Jacques, 2008; Falardeau et al., 2013) and non-peptide (Hamdache et al., 2011; Wise et al., 2012) antimicrobial compounds. Among the most studied of these antimicrobials are the three cyclic lipopeptide families surfactin, iturin, and fengycin (Ongena and Jacques, 2008; Falardeau et al., 2013). Conversely, less is known about *B. linens* antimicrobials. *B. linens* has been found to produce an antibacterial peptide (Motta and Brandelli, 2002) as well as the antifungal compound methanethiol (Kerr, 1999). When *B. subtilis* was further investigated for its potential antifungal compounds, it was revealed that it produced two variants of the antimicrobial cyclic lipopeptide surfactin (Pecci et al., 2010; Falardeau et al., 2013). Surfactin is produced by many antagonistic *Bacillus* spp., including *B. subtilis*, and is reported to provide antimicrobial effects by pore formation in cell membranes. While surfactins are generally thought to be more inhibitory against bacterial cells and to possess less antifungal activity (Pérez-García et al. 2011), other work has shown their antifungal effects on various fungal cells (Chan et al., 2009; Cao et al. 2012; Rebib et al. 2012).

Although *B. linens* IC10 and *B. subtilis* had similar *in vitro* efficacy against both fungal mycelia, their activity was much different in suppressing tomato fruit diseases. Indeed, the bacteria were much more effective in controlling *B. cinerea* on tomato whereas they were much less effective against *A. solani*. This was also apparent when assessing antifungal compounds in the crude bacterial extracts. Again, bacterial extracts showed similar antifungal effects against mycelial growth and spore germination of both fungi, whereas they were much less effective against *A. solani in vivo*. This may indicate that mechanisms in addition to antibiosis might be involved in the increased suppressive effects of the bacteria toward *B. cinerea*, such as induction of plant defense mechanisms in the tomato (Zhang et al., 1998; Haggag and Saber, 2007; Siddiqui et al., 2009).

Overall, results from this study showed that bacteria isolated from suppressive compost tea inhibited fungal growth *in vitro* and disease *in vivo*. The two most effective bacteria were often found to function in synergy. Antifungal compounds, including surfactins, were found in the bacterial extracts suggesting that antibiosis is a main mechanism of action. However, mode of action involving induction of tomato defense responses may also be involved. Future work will attempt (i) to profile and identify microbial antagonist populations by PCR-DGGE and pyrosequencing in highly suppressive compost teas and (ii) to identify compost tea preparation parameters that enhance populations of these antagonist bacteria as well as (iii) to identify additional antifungal compounds and elucidate their modes of action.

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