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Identification and use of potential bacterial organic antifungal volatiles in biocontrol

W.G. Dilantha Fernando[a,*], Rajesh Ramarathnam[a], Akkanas S. Krishnamoorthy[b], Sarah C. Savchuk[a]

[a]*Department of Plant Science, University of Manitoba, 222 Agriculture Building, 66 Dafoe Road, Winnipeg, MB., Canada R3T 2N2*
[b]*Department of Plant Pathology, Tamilnadu Agriculture University, Coimbatore, Tamilnadu 641 003, India*

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Abstract

Bacteria, isolated from canola and soybean plants, produced antifungal organic volatile compounds. These compounds inhibited sclerotia and ascospore germination, and mycelial growth of *Sclerotinia sclerotiorum*, in vitro and in soil tests. Ascospore germination in cavity slides was inhibited 54–90% by the volatile producers. When mycelial plugs or the sclerotia, exposed to these volatiles, were transferred to fresh agar plates, the pathogen could not grow, indicating the fungicidal nature of the volatiles. Head space volatiles, produced by bacteria, were trapped with activated charcoal, by passing nitrogen continuously over shake cultures for 48 h. The compounds were eluted from the charcoal with methylene chloride and identified using Gas Chromatography–Mass Spectrometry (GC–MS). The volatile compounds included aldehydes, alcohols, ketones and sulfides. Of the 23 compounds assayed for antifungal activity in divided Petri plates, with filter-disks soaked with these compounds (100 and 150 μl), only six compounds completely inhibited mycelial growth or sclerotia formation, suggesting their potential role in biological control. The compounds are benzothiazole, cyclohexanol, *n*-decanal, dimethyl trisulfide, 2-ethyl 1-hexanol, and nonanal. Volatiles may play an important role in the inhibition of sclerotial activity, limiting ascospore production, and reducing disease levels. Studies are under way to understand this phenomenon under field conditions. This is the first report on the identification and use of bacterial antifungal organic volatiles in biocontrol.

Keywords: Bacteria; Organic volatiles; *Sclerotinia sclerotiorum*; Biocontrol

1. Introduction

Biological control of plant pathogens involves harnessing disease-suppressive microorganisms to improve plant health (Handelsman and Stabb, 1996). Biocontrol seems to be a reliable alternative to chemical fungicides, which have raised serious concerns of food contamination and environmental pollution. Biocontrol is eco-friendly, safe and may provide long-term protection to the crop. Some saprotrophic bacteria, can serve as excellent biocontrol agents against plant pathogens. *Pseudomonas* spp. produce secondary metabolites such as antibiotics or pyoverdine siderophores (Loper and Buyer, 1991), or compete for nutrients (Andrews, 1992) suppressing plant pathogens. Pseudomonads produce a wide range of antibiotics, which includes phenazine (Thomashow and Weller, 1988), pyrrolnitrin (Kanner et al., 1978), oomycin A (Howell and Stipanovic, 1979), geldanamycin (Rothrock and Gottlieb, 1984), pyoluteorin (James and Gutterson, 1986), pyocyanin (Gutterson et al., 1988) and 2-4 diacetylphloroglucinol (Vincent et al., 1991). *Pseudomonas* spp. are also capable of producing organic volatiles, whose in vitro antifungal nature has been demonstrated against *Phytophthora vignae* in cowpea (Fernando and Linderman, 1994). But to our knowledge, there has been no work initiated on the use of organic volatiles, produced by bacterial antagonists, in the biocontrol of plant diseases. Work was initiated in our lab to investigate the role of bacterial organic volatiles in biocontrol of plant pathogens. For this purpose, a pathogen

* Corresponding author. Tel.: +1 204 474 6072; fax: +1 204 474 7528.
E-mail address: d_fernando@umanitoba.ca (W.G.D. Fernando).

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with a wide host range was selected. *Sclerotinia sclerotiorum* (Lib.) de Bary causes disease conditions on over 400 crops species. None of these crops has significant genetic resistance to the pathogen and biocontrol would be a useful alternative to chemical control.

S. sclerotiorum causes some of the most economically important diseases of western Canada, including stem rot of canola, head, stem and basal rot of sunflower, white mold of beans, and crown and stem rot of alfalfa (Martens et al., 1994). Biological control of *S. sclerotiorum* has been successfully demonstrated in numerous studies over the past 20 years, through oxalate oxidase (Tu, 1985), hyperparasitism (Huang and Kokko, 1993; Gracia-Garza et al., 1997), nutrient competition (Yuen, 1991) and antibiosis (Oedjijono et al., 1993). However, the majority of the work initiated so far has concentrated on the use of fungal mycoparasites such as *Coniothyrium minitans* that degrade the sclerotia in the soil (Gerlagh et al., 1999). There is limited research on the use of bacterial antagonists of *S. sclerotiorum*, other than the reports on inhibition of ascospore germination on petals under field conditions (Savchuk, 2002) and inhibition of apothecial formation (Thaning et al., 2001).

Diseases caused by *S. sclerotiorum* can be effectively controlled if the overwintering sclerotia, the primary inoculum producers, can be destroyed, thus preventing mycelial germination, apothecial formation and ascospore release. Bacterial antifungal volatiles may have the potential, as they can diffuse through the soil and kill the overwintering sclerotia. The objectives of this study were to: (1) identify antifungal volatile-producing bacteria isolated from canola and soybean plants; (2) determine the target of inhibition, such as mycelial growth, sclerotial and ascospore germination, both in vitro and in soil; (3) isolate and identify the volatiles through Gas Chromatography–Mass Spectrometry; (4) screen individual organic volatile compounds for the inhibition of mycelial growth and sclerotial germination.

2. Materials and methods

2.1. Bacterial strains, fungal strain and culture conditions

The bacterial strains used in this study are listed in Table 1. The bacterial stock cultures were maintained in Luria Bertani broth (LBB), amended with 20% glycerol and stored at −80 °C. Plate cultures were started by streaking the bacteria from the stock onto Luria Bertani agar (LBA) or nutrient agar ((NA) Difco Laboratories, Detroit, MI) plates and incubated at 28 °C for 24 h. Tryptic soy agar ((TSA) Difco Laboratories, Detroit, MI) and NA were used for the volatile assays. Bacterial cultures for the volatile assays were grown in tryptic soy broth (TSB) at 28 °C for 16–18 h and shaken at 180 rpm in an incubator shaker (Jeio Tech SI-600, Seoul city, South Korea). The fungal strain *S. sclerotiorum* SS33, which had the most consistent high virulence, was used in all the experiments. Fresh cultures were started by placing surface sterilized, cut sclerotia in the middle of a potato dextrose agar ((PDA) Difco Laboratories, Detroit, MI) plate. The plates were incubated at room temperature for 7–8 d, until mycelial growth led to the formation of new sclerotia.

Table 1
Bacterial strains and their origin

Isolate #	Name of the strain	Origin
DF29	*Pseudomonas fluorescens* (Biotype-G)	Canola stem
DF33	*Pseudomonas fluorescens* (Biotype-G)	Canola stem
DF35	*Pseudomonas fluorescens* (Biotype-G)	Canola root tip
DF41	*Pseudomonas corrugata*	Canola root tip
PA-23	*Pseudomonas chlororaphis* (Biotype-D)	Bean Field
DF190	*Pseudomonas chlororaphis* (Biotype-D)	Canola stubble from surface
DF191	–	Canola stubble from surface
DF199	–	Canola stubble from 5 cm depth
DF200	*Pseudomonas aurantiaca*	Canola stubble from 5 cm depth
DF202	*Pseudomonas chlororaphis* (Biotype-D)	Canola stubble from 5 cm depth
DF209	*Pseudomonas chlororaphis* (Biotype-D)	Canola stubble from 10 cm depth
DF210	*Pseudomonas chlororaphis* (Biotype-D)	Canola stubble from 10 cm depth
DF220	–	Canola stubble from sub-surface
DF223	–	Canola stubble from 5 cm depth

2.2. Screening for antifungal volatile producing bacteria

Antifungal volatile activity was identified using a modification of the methods described by Fernando and Linderman (1994). A total of 197 bacterial isolates were streaked onto one half of a divided plate containing TSA and the plates were immediately wrapped in Parafilm® (Pechinery Plastic Packaging, Menasha, WI) to seal in the volatiles. Following a 24 h incubation period at 25 °C, 5 mm mycelial plugs of *S. sclerotiorum* were placed on the other half of the divided plate, containing PDA, and the plates re-sealed. Measurements of radial mycelial growth were taken 72 h post-inoculation of the pathogen, on both bacterial and control plates. In the initial screening there were two replicates per isolate. Those isolates providing >75% mycelial inhibition were screened again in a four-replicate experiment.

2.3. Biolog identification of bacteria

Bacteria demonstrating consistent antifungal volatile activity were selected for further identification, using

the Microlog™ system (Biolog, Inc., Hayward, CA). Single colonies were obtained by streaking on media and the following steps were performed in the process of identification: (i) Gram stain (Biolog, Inc.) rated as Gram positive or Gram negative; (ii) bacteria were streaked onto Biolog universal growth (BUG) agar medium (Biolog, Inc.); (iii) approximate bacterial number was quantified with a turbidimeter, and 150 µl of the bacterial solution were pipetted into each of the 96 wells in the Biolog microplates; (iv) the plates were incubated at 32 °C for 16–24 h and then read with an automated plate reader (Biolog, Inc.), assessed visually and identified to genus or species level.

2.4. Bacterial volatile production—divided plate method

A three-compartment Petri plate (85 mm diam.) was used. Two experimental designs were employed. In the first design, the first compartment, containing NA or TSA, was streaked with the bacterial strain, a second compartment containing PDA was inoculated in the center with a 5 mm mycelial disc of *S. sclerotiorum* cut from the margin of an actively growing culture, and the third compartment was left empty. In the second design, the third compartment was filled with activated charcoal to adsorb the volatiles produced by the bacteria and to check the effect on mycelial growth. The plates were sealed tightly after inoculation and further incubated at 25 °C for 7 d. The growth of the fungus was measured every 24 h, as compared to the control, over a period of 7 d. The control plates had only the mycelial discs growing in them. The setup described above was also used to check for the inhibition of germination of surface sterilized sclerotia. After 7 d, the mycelial plugs and sclerotia from all the plates were removed and tested for viability in a fresh Petri plate containing PDA. There were four replicates for each treatment and the experiments were repeated twice.

2.5. Bacterial volatile production—sealed plate method

Bacteria were streaked on to NA or TSA in the bottom dish of a Petri plate. A 5 mm mycelial plug was cut from the margin of an actively growing culture and placed in the centre of the bottom dish of a second Petri plate containing PDA. The dish containing the mycelial plug was inverted over the bacterial plate and the dishes were sealed with Parafilm. The plates were incubated at room temperature. The growth of the fungus was measured every 24 h, as compared to the control, over a period of 7 d. The control plates had only the mycelial discs growing in them. The setup described above, was also used to check for the inhibition of surface sterilized sclerotial germination. After 7 d, all mycelial plugs and sclerotia were removed from the plates and tested for viability in a fresh Petri plate containing PDA. There were four replicates for each treatment and the experiments were repeated twice.

2.6. Bacterial volatile production in soil

Mycelial and sclerotial inhibition was also tested in soil. Seven to eight grams of autoclaved field soil or Vermiculite, placed in the bottom dish of a Petri plate, was mixed with 30–35 ml bacterial culture (10^8 CFU/ml) that had been incubated for 16–18 h in TSB. Otherwise the experimental setup was the same as described for the bacterial volatile production—sealed plate method. The ability of the volatiles to penetrate through soil was also tested. Ten surface sterilized sclerotia were packed in nylon mesh (Windsor Plywood, Winnipeg, Man., Canada) and placed at 1 cm depth in sterile field soil or Vermiculite contained in 150 mm sterile glass Petri plates. Bacteria were streaked onto TSA in an identical bottom dish of a second150 mm sterile glass Petri plate, which was inverted over the soil-containing dish. The plates were sealed together with Parafilm and incubated at room temperature. The control plates had no bacteria. The buried sclerotia were removed after 10 d, replated on PDA and checked for germination. There were four replicates for each treatment and the experiments were repeated once.

2.7. Bacterial volatile production—ascospore inhibition

Ascospores (5×10^4 spores/ml) of *S. sclerotiorum* in phosphate buffer (pH 7.0) were placed in a cavity slide. The slide was then kept inside the bottom dish of a sterile Petri plate. The bottom dish of a second Petri plate became the top lid containing the bacterial culture on TSA. The two dishes were sealed together to allow the volatiles to act on the ascospores. After 24, 48 and 72 h, the slides were observed for spore germination under a microscope. Five microscopic fields were selected ($10\times45\times$) and in each field 10 ascospores were observed. The ascospores were considered germinated when the length of the germ tube was twice the size of the ascospore. The percentage inhibition of spore germination was calculated as compared to the control. There were four replicates for each treatment and the experiments were repeated once.

2.8. Collection of volatile organic compounds

Headspace volatiles produced by the bacteria were collected using a setup, with slight modifications recommended by DeMilo et al. (1996). The bacterial isolates were shake-cultured in 100 ml of TSB contained in 250 ml conical flasks, each fitted with a two-way rubber cork with glass tubes inserted through it. One of the inserted glass tubes was placed just 1 cm above the bacterial culture and the other end of the tube was connected to a nitrogen supply system for removing the headspace volatiles. One end of the second tube was positioned near the neck of the flask and the other end connected to a volatile trap. The volatile trap was made of glass tube (7 cm length and 0.4 cm diam) containing 150 mg of activated charcoal

(Darco®, 20–40 mesh, Aldrich, Milwaukee, WI). Before use, the traps were placed in a 150 mm Petri plate, which was wrapped in aluminum foil, and sterilized in a 350 °C oven for 24–36 h. The neck of the conical flask was tightly sealed with Parafilm to prevent the escape of volatile organic compounds. TSB, with no bacteria, was used as the control. The stream of dry nitrogen flow was started 12 h post-inoculation of the bacteria and maintained at 300 ml/min for up to 48 h. Flasks were shaken throughout the collection process. The volatiles in the activated charcoal trap were eluted into glass vials, with 0.5 ml of methylene chloride.

2.9. GC–MS analysis of the volatiles

Gas chromatography was performed using a Varian Star 3400 CX series GC with a flame ionization detector, available in the Department of Chemistry, University of Manitoba, Winnipeg. The samples were separated on a 15 m DB-1 megabore column of 100% dimethylpolysiloxane (Shojania et al., 1999). The trap contents were absorbed at 22 °C from a unijector (SGE) on to a fused silica column (BP1, 25 m length × 0.22 mm i.d., 0.25 μm film thickness), which was cryogenically focused with dry ice and acetone for 2 min. The column was attached to a Hewlett-Packard 5890 Gas Chromatograph attached to a Hewlett-Packard mass selective detector. Helium was used as the carrier gas and the flow rate was maintained at 1 ml/min. Column temperatures were programmed from 30 to 200 °C at 4 °C/min ramp rate. The mass spectra of the unknown compounds were compared with those in the NIST/EPA/NIH Mass Spec. Library (Version 2.0).

2.10. Analysis of antifungal activity of organic volatile compounds

The compounds identified through GC–MS analysis were purchased (synthetic chemicals) to carry out further antifungal tests. Each of the compounds was tested for antifungal activity by placing 100 and 150 μl of each compound, singly and in combination with TSB, on sterile filter paper discs placed on TSA in divided agar plates. Fungal mycelial plugs or sclerotia were placed on the PDA side of the divided plate. Some of the compounds were identified as being present in both the bacteria treatment and the control (TSB) in GC–MS analysis. The compounds were tested in combination with TSB to check the influence of the broth on the antifungal activity of the compounds.

2.11. Data analysis

Experiments were analyzed using analysis of variance (ANOVA), and a mean separation test (Fisher's least significant difference) was performed at $P = 0.05$, using the Analyst procedure of SAS, Version 8.1 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Screening for antifungal volatile producing bacteria

Of the 197 isolates screened for the production of volatile antifungal compounds in divided plates, 14 isolates consistently produced antifungal volatiles, and inhibited mycelial growth and sclerotial germination of *S. sclerotiorum*. The mycelial plugs and the sclerotia from these bacterial treatments failed to germinate, when plated on fresh PDA plates. These 14 isolates were considered for further experiments.

3.2. Biolog identification of bacteria

Of the 14 isolates run through the Biolog procedure for identification, 10 isolates were identified to the species level. Of the 10 isolates, five *Pseudomonas chlororaphis* (Biotype D), three *Pseudomonas fluorescens* (Biotype G), one *Pseudomonas corrugata* and one *Pseudomonas aurantiaca* were identified (Table 1).

3.3. Bacterial volatile production—divided plate method

Antifungal volatile activity was observed in both NA and TSA divided plates. In plates amended with the activated charcoal in the third compartment, none of the bacteria had any inhibitory effect, allowing complete growth of the mycelial plug and germination of the sclerotia (Fig. 1). Bacterial growth on TSA plates had more antifungal volatile activity, and mycelial and sclerotial inhibition than on NA plates (Fig. 2). Of the 14 isolates screened for the production of antifungal volatiles, 12 isolates completely inhibited the mycelial growth and sclerotial germination. Data of inhibition in divided plates mediated by five isolates; DF35 isolated from canola root tip, PA-23 from soybean, DF190 from the surface stubble, DF200 from stubble at 5 cm depth and DF209 isolated from stubble at 10 cm depth are presented in Fig. 2. These bacteria were selected based on their origin from the soil environment for volatile mediated suppression of *S. sclerotiorum*, and also as representative of their respective groups of origin. In the NA divided plate assays, all the isolates caused higher inhibition of the sclerotial germination than inhibition of the mycelial germination (Fig. 2A and C). Isolate DF35 was able to inhibit sclerotial germination by 100%, but the mycelial growth only by 50%, while the other bacteria caused 100% inhibition of both the growth of the mycelia and germination of the sclerotia (Fig. 2B and D). All bacterial mediated inhibition of mycelial growth and sclerotial germination in TSA plates were significantly different from that of the control. Mycelial plugs and sclerotia in the control plates grew without any inhibition. Mycelial plugs and sclerotia replated on fresh PDA plates from the inhibitory bacterial treatment plates, failed to grow.

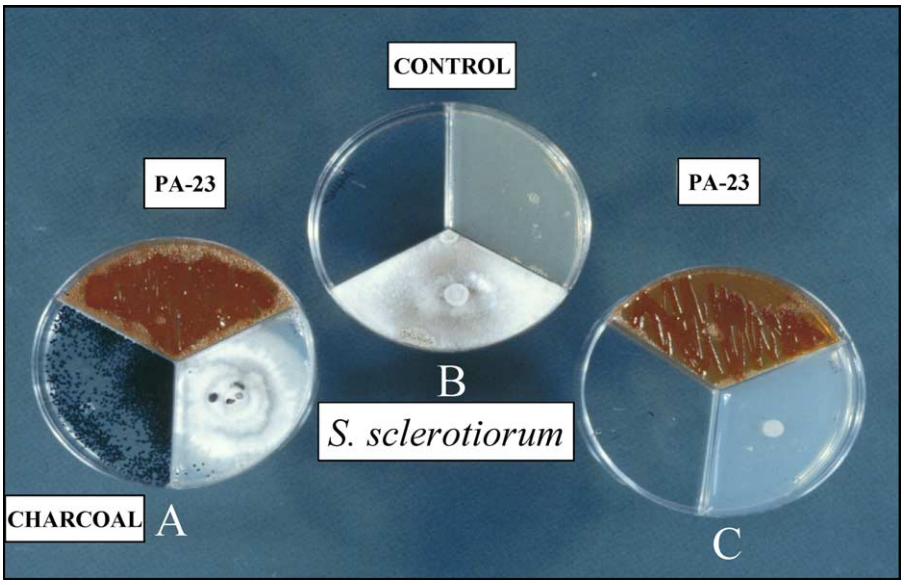

Fig. 1. Bacterial antifungal-volatile activity in divided plates. Mycelial plug growth was completely inhibited in the presence of the bacteria streaked in a different compartment (C), as compared to the control (B), which had no bacteria. Mycelial growth was unaffected by the presence of volatile producing bacteria, when the third compartment of the plates was amended with activated charcoal (A). Charcoal adsorbs volatiles as soon as they are produced, and hence no inhibitory effect was observed.

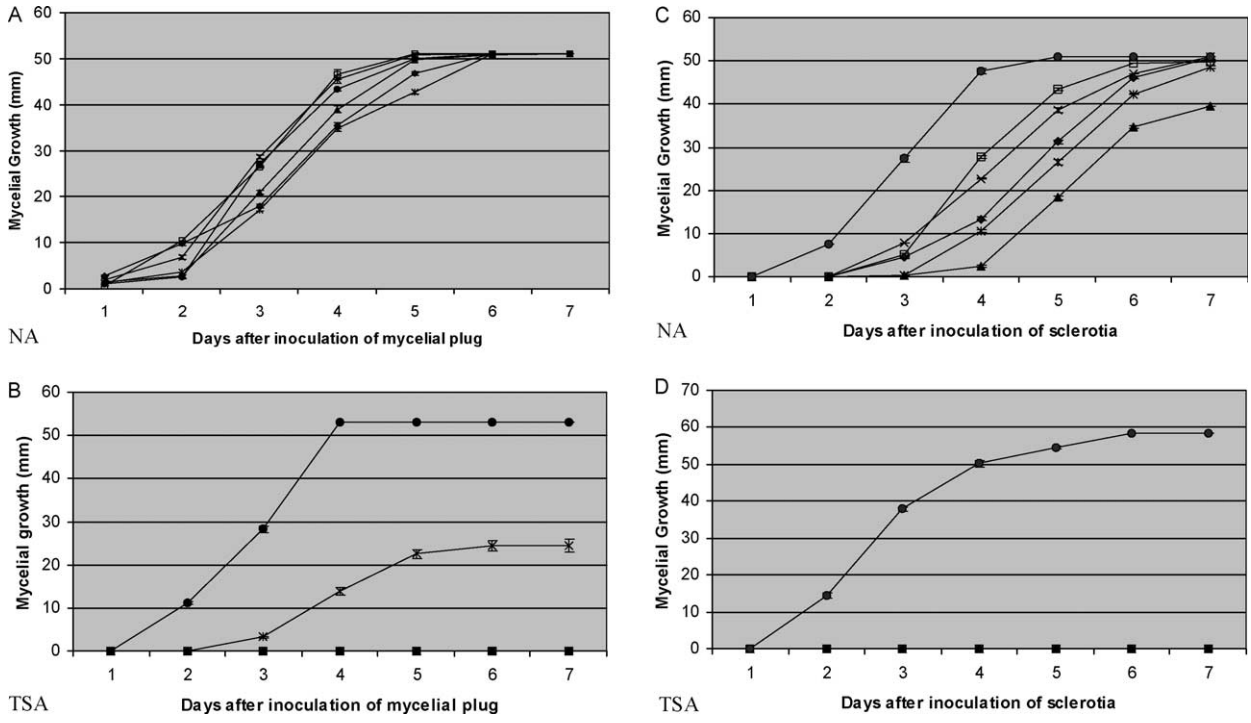

Fig. 2. Bacterial antifungal-volatile assays (divided plates). (A) Mycelial inhibition on NA/PDA. (B) Mycelial inhibition on TSA/PDA. (C) Sclerotial inhibition on NA/PDA. (D) Sclerotial inhibition on TSA/PDA. Bacterial volatile production was enhanced by TSA (B and D), resulting in higher inhibition of mycelial growth and sclerotial germination on TSA than on NA (A and C). Also, in the NA plates, the inhibition of the sclerotial germination was higher than the mycelial inhibition. In the NA plates, mycelial growth in bacterial treatments were significantly different from the control up to 5 days post-inoculation of the mycelial plug (Fisher's LSD = 1.06 mm) and up to 6 days post-inoculation of sclerotia (Fisher's LSD = 0.78 mm). Of the five isolates presented in the figure, isolates, PA-23 (X's), DF190 (filled diamond), DF200 (square), DF209 (Filled triangle) had 100% inhibition of the mycelial plug (Fisher's LSD = 1.08 mm) and the sclerotia (Fisher's LSD = 0.56 mm) on TSA, as compared to the control (filled circle). Isolates PA-23, DF190, DF200 and DF209 are seen at the bottom of the graph (0 mm) in (B), due to their 100% inhibition of the mycelial growth on TSA. Isolate DF35 (asterisk) showed 50% inhibition of mycelial growth and 100% inhibition of sclerotial germination in TSA divided plates. All the bacterial treatments are seen at the bottom of the graph (0 mm) in (D), due to their 100% inhibition of sclerotial germination on TSA. The values used are the mean of the radial mycelial growth of *S. sclerotiorum*. Each treatment had four replicates and the experiments were done twice. Bars represent the standard error of mean of eight replicates.

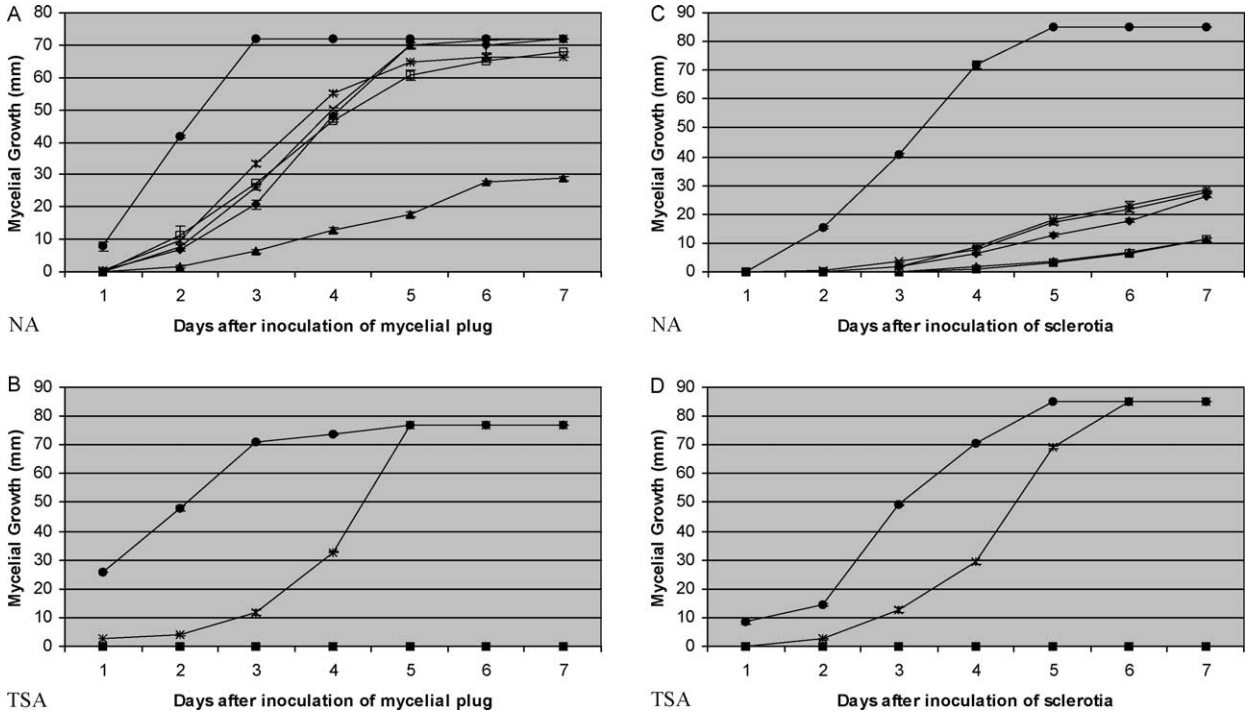

Fig. 3. Bacterial antifungal-volatile assays (sealed plates). (A) Mycelial inhibition on NA/PDA. (B) Mycelial inhibition on TSA/PDA. (C) Sclerotial inhibition on NA/PDA. (D) Sclerotial inhibition on TSA/PDA. Of the five isolates presented in the figure, isolates, PA-23 (X's), DF190 (filled diamond), DF200 (square), DF209 (filled triangle) had 100% inhibition of the mycelial plug (Fisher's LSD = 0.58 mm) and the sclerotia (Fisher's LSD = 0.66 mm) on TSA, as compared to the control (filled circle). Bacterial treatments had significantly higher inhibition of the sclerotial germination (Fisher's LSD = 1.53 mm) than the mycelial growth (Fisher's LSD = 2.22 mm) on NA plates. Isolates PA-23, DF190, DF200 and DF209 are seen at the bottom (0 mm) of (B and D), due to their 100% inhibition of both mycelial and sclerotial germination on TSA. Values used are the mean of the radial mycelial growth of *S. sclerotiorum*. Each treatment had four replicates and the experiments were done twice. Bars represent the standard error of mean of eight replicates.

3.4. Bacterial volatile production—sealed plate method

Tryptic soy agar enhanced antifungal volatile production, and inhibition of the mycelial growth and sclerotial production in all the 14 isolates tested. Data of inhibition in sealed plates mediated by five isolates, DF35, PA-23, DF190, DF200 and DF209 are presented in Fig. 3. In the NA sealed plate assays, all the bacteria had higher inhibition of sclerotial germination than mycelial germination, except isolate DF209 that had high inhibition of both (Fig. 3A and C). Control plates had normal mycelial growth and sclerotial germination. All isolates had complete inhibition of both mycelial and sclerotial germination on TSA, except isolate DF35 that had mycelial growth and sclerotial germination same as the control at 5 and 6 days after inoculation, respectively (Fig. 3B and D). Mycelial plugs and sclerotia from the inhibitory bacterial treatment plates failed to grow when replated on fresh PDA.

3.5. Bacterial volatile production in soil

In the soil assays, when soil was amended with TSB grown bacteria, all 14 bacterial treatments showed inhibition of mycelial growth and sclerotial germination. Mycelial plugs and sclerotia following treatment failed to grow and germinate when replated on fresh PDA plates. In the sclerotia burial experiments, of the 14 bacteria tested, significant control was observed only with bacterial isolates DF200 and DF209 in the soil.

3.6. Bacterial volatile production—ascospore inhibition

Of the 14 bacteria tested, isolates DF200 and DF209 had high inhibition of ascospore germination at 88 and 90%, respectively; isolate DF190 had an intermediate inhibition at 54%; isolates DF35 and PA-23 had the least inhibition percentage at 32 and 34%, respectively.

3.7. GC–MS analysis of the volatiles

Fourteen bacteria were subjected to the GC–MS analysis. The GC–MS analysis of volatile compounds eluted from charcoal traps and subsequent database search yielded 23 organic compounds, which included a range of aldehydes, ketones, alcohols, aliphatic alkanes, organic acids, etc. (Table 2). Organic volatile compounds, with similarity index (SI) > 850 from the database search (Fig. 4), were analyzed through antifungal activity assays in divided plates.

Table 2
Test for inhibition of mycelial plug/sclerotia by volatile organic compounds identified from GC–MS analysis

Compound	Inhibition of mycelial plug/sclerotia	Sclerotial formation	Viability when replated
Cyclohexanol	Yes	−	No
Cyclohexanol + TSB	Yes	−	No
Decanal	Yes	−	No
Decanal + TSB	Yes	−	No
2-Ethyl,1-hexanaol	Yes	−	No
2-Ethyl,1-hexanaol + TSB	Yes	−	No
Nonanal	Yes	−	No
Nonanal + TSB	Yes	−	No
Benzothiazole	Yes	−	No
Benzothiazole + TSB	Yes	−	No
Dimethyl trisulfide	Yes	−	No
Dimethyl trisulfide + TSB	Yes	−	No
Dodecane	No	+*	Yes
Dodecane + TSB	No	+*	Yes
Undecane	No	+*	Yes
Undecane + TSB	No	+*	Yes
Nonane	No	+*	Yes
Nonane + TSB	No	+*	Yes
Decane	No	+*	Yes
Decane + TSB	No	+*	Yes
Nonadecane	No	+	Yes
Nonadecane + TSB	No	+	Yes
1-Heptedecanol	No	+	Yes
1-Heptedecanol + TSB	No	+	Yes
4-Octybenzoic acid	No	+	Yes
4-Octybenzoic acid + TSB	No	+	Yes
Phenylenediamine	No	+	Yes
Phenylenediamine + TSB	No	+	Yes
2-Methyl pyrazine	No	+	Yes
2-Methyl pyrazine + TSB	No	+	Yes
Benzaldehyde	No	+	Yes
Benzaldehyde + TSB	No	+	Yes
Hexadecane	No	+	Yes
Hexadecane + TSB	No	+	Yes
Pyrazine	No	+	Yes
Pyrazine + TSB	No	+	Yes
Tetradecane	No	+	Yes
Tetradecane + TSB	No	+	Yes
Pentadecane	No	+	Yes
Pentadecane + TSB	No	+	Yes
1-Undecene	No	+	Yes
1-Undecene + TSB	No	+	Yes
2-Undecanone	No	+	Yes
2-Undecanone + TSB	No	+	Yes
2-Tridecanone	No	+	Yes
2-Tridecanone + TSB	No	+	Yes

The 23 organic volatile compounds identified through GC/MS, were individually and in combination with TSB, tested for the inhibition of germination of mycelial plugs and sclerotia. TSB was tested to check for its synergistic effect with the compounds towards mycelial and sclerotial inhibition. Of the 23 tested, only six compounds, cyclohexanal, decanal, 2-ethyl, 1-hexanol, nonanal, benzothiazole and dimethyl trisulfide, inhibited the germination of the mycelial plug and the sclerotia at 100 and 150 μl quantity dispensed. The mycelial plugs and the sclerotia from these plates did not grow, when replated on fresh PDA, while those from the other treatments did. The other treatment plates also had sclerotial formation following the normal mycelial growth. * The sclerotia formed in the plates of dodecane, undecane, nonane, and their combination with TSB, were oddly shaped and soft to touch, but had normal growth when replated on fresh PDA.

3.8. Analysis of antifungal activity of organic volatile compounds

Of the 23 compounds tested, only six completely inhibited mycelial growth and sclerotial germination at both tested volumes of 100 and 150 μl. The antifungal compounds are listed in Table 3 (Fig. 5). Sclerotia from plates of Undecane, *n*-Decane, Dodecane, and *n*-Nonane were abnormally shaped and very spongy when touched. However, when they were plated on fresh PDA plates, normal germination was observed. Fungal mycelial plugs or sclerotia from the antifungal volatile plates, when replated on fresh PDA plates, failed to grow. Benzaldehyde and pyrazine derivatives showed up in both the control and bacterial-treatment GC runs. Therefore, the compounds were combined with TSB and loaded on discs to test the effect of TSB on the activity of the compounds. TSB had neither positive nor negative effect on the antifungal activity of any of the 23 compounds tested.

4. Discussion

Bacteria isolated from canola root and stubble and from soybean roots produced volatiles that were inhibitory to survival (sclerotia), infection (mycelia) and reproductive structures (ascospores) of *S. sclerotiorum*. Of the 14 bacteria tested, 12 isolates that had medium-to-high levels of antifungal volatile production were isolated from the soil and soil-associated plant parts. This is an interesting phenomenon; if volatile antibiotics were to be effective in controlling the pathogen the most effective place would be in the soil, where these volatiles would come in direct contact with the sclerotinia propagules. On the other hand, the oxalate oxidase producing bacteria, e.g. *Pantoea agglomerans*, were isolated from leaves and flowers of canola (Savchuk and Fernando, unpublished). If oxalic acid, a pathogenicity factor produced by *S. sclerotiorum*, is to be degraded by oxalate oxidase, the bacteria capable of producing the enzyme should be present on leaves and flowers to be most effective. The volatile producing bacteria, *Pseudomonas* sp. are common rhizosphere bacteria, while the *P. agglomerans* are common epiphytic phyllosphere bacteria. Carbon (Fiddaman and Rossall, 1994) and nitrogen sources seem to increase volatile production, as seen in this study, where TSA, richer in carbon and nitrogen sources than NA, seems to induce more volatile production and antifungal activity. This is in support of the earlier known fact that volatile production in the soil is high in the organic niches, such as stubble, which are the carbon and nitrogen rich sources for the microflora and hence the sites of competition for nutrients. The antifungal activity of the volatiles has been clearly demonstrated in this study, when the presence of the activated charcoal, which adsorbed the volatiles as soon as the bacteria produced them, inhibited all antifungal activity. The main mode of action of these antifungal volatiles is their

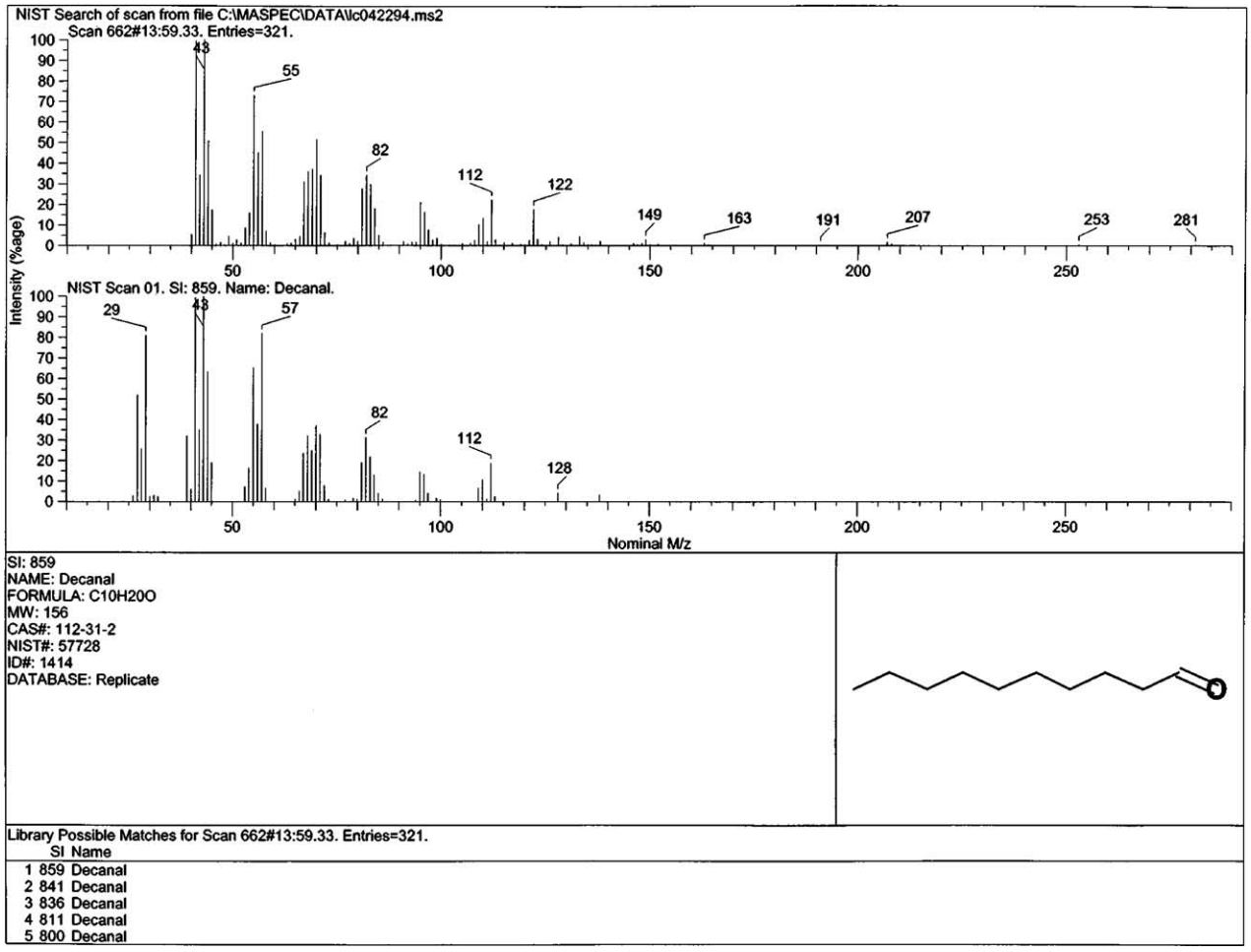

Fig. 4. GC–MS data analysis of *n*-decanal. The GC–MS analysis and the subsequent database search yielded 23 organic volatile compounds. Compounds, with similarity index (SI) >850 from the database search, were considered for the in vitro antifungal assays in divided plates.

fungicidal effect on the sclerotia, which prevents the sclerotia from germinating even under favorable conditions, e.g. on fresh PDA plates after the bacterial treatment. The control of the sclerotia is key in the control of *S. sclerotiorum*, as sclerotial control would reduce the apothecial formation, which would decimate ascospore production, the most important infection units in many crops, and would consequently reduce several diseases (Abawi and Grogan, 1979). The bacteria not only produced the volatiles in vitro but also in the soil, as demonstrated in the sealed plates, where soil amended with bacteria was inhibitory to fungal growth. The experiments also proved the ability of the volatiles in soil and their capacity to prevent sclerotial germination. This might indicate the use of these bacteria as soil amendments for the management of overwintering

Table 3
Antifungal volatiles and their bacterial origin

Name of the compound	Bacterial strain
Nonanal	DF200, DF202, DF209, DF220, PA-23
Cyclohexanol	DF209
Benzothiazole	DF220, PA-23
2-Ethyl, 1-hexanol	DF29, DF33, DF35, DF41, DF190, DF199, DF200, DF202, DF209, DF220, DF223, PA-23
n-Decanal	DF200, DF202, DF 220
Dimethyl trisulfide	DF199, DF202

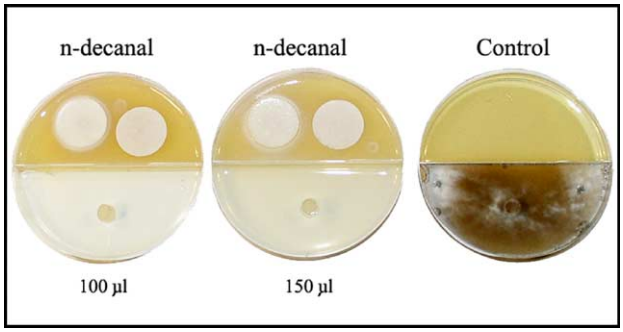

Fig. 5. Antifungal activity of *n*-decanal in divided plates. The compounds, identified through GC–MS analysis, used in this assay are synthetic chemicals that were purchased. Each of the compounds was tested for antifungal activity by placing 100 and 150 µl of each compound, singly and in combination with tryptic soy broth (TSB), on sterile filter paper discs placed on TSA in divided agar plates. TSB did not have any positive or negative effect on the antifungal activity of any of the 23 compounds tested.

structures in soil. Pathogen-infested stubble and sclerotia are substrates for the production of sexual and asexual spores that cause primary infection of the crop. Destruction of the overwintering structures would greatly limit primary inoculum production and establishment of the disease in the crop.

The antifungal nature of the organic volatiles has been demonstrated in several pathogen systems, such as inhibition of hyphal extension and formation of arthrospore in *Geotrichum candidum* by trimethylamine (Robinson et al., 1989), inhibition of carpogenic germination of sclerotia of *S. sclerotiorum* in bean by allyl alcohol (Huang et al., 1997), and control of root rot of tobacco by hydrogen cyanide produced by pseudomonads (Voisard et al., 1989). Other than organic volatiles, inorganic volatiles such as ammonia, produced by *Enterobacter cloacae*, appear to be one of many mechanisms that bacteria use in the biocontrol of pre-emergence damping-off caused by *Pythium* spp. (Howell et al., 1988). Fungal response to bacterial volatiles appears to be species-, environment-, and age-specific (Mackie and Wheatley, 1999). Allyl alcohol inhibits carpogenic germination of sclerotia of *S. sclerotiorum*, but at the same time stimulates growth and enhances sclerotial colonization by *Trichoderma* spp. (Huang et al., 1997). Allyl alcohol also increases populations of beneficial bacteria such as *P. fluorescens* and *P. putida* (Domsch, 1959; Altman and Lawlor, 1966). The chemical nature of the organic volatiles appears to determine their antifungal activity. Aliphatic aldehydes were more effective in the post-harvest control of gray mould caused by *Botrytis cinerea* in strawberry, blackberry and grape (Archibold et al., 1997), and aliphatic aldehydes and ketones were more effective than alcohols in the inhibition of germ tube formation of *Alternaria alternata* (Andersen et al., 1994). In our study, of the six bacterial volatiles with high fungicidal activity, two were aldehydes (nonanal and *n*-decanal), and two were alcohols (cyclohexanol and 2-ethyl, 1-hexanol) (Table 3). Two sulfur-based compounds, benzothiazole and dimethyl trisulfide, also showed high fungicidal activity. Many commercially used fungicides and soil fumigants are also sulfur-based. The antifungal activity of nonanal and decanal has been demonstrated earlier. Nonanal and decanal are the major constituents of the essential oil of *Hibiscus cannabinus*, and responsible for the fungitoxic activity of the oil towards three species of *Colletotrichum* (Kobaisy et al., 2001). Nonanal, produced by cotton leaves, is responsible for the production of the unique aerial hyphae and decrease in the aflatoxin production of *Aspergillus flavus* and *A. parasiticus* (Green- McDowelle et al., 1999). Our data indicate that DF200 (*P. aurantiaca*) and DF209 (*P. chlororaphis* Biotype D), which were capable of inhibiting sclerotia buried in the soil, produced nonanal, and DF200 also produced decanal. The presence of the α, β-unsaturated bond adjacent to the carbonyl moiety makes the molecule more reactive and enhances antifungal activity, as seen in (*E*) 2-nonenal, (*E*) 2-hexenal, which are more antifungal than nonanal (Andersen et al., 1994). These compounds, especially (*E*) 2-hexenal, are major constituents of plant volatiles that are produced during wounding, through the lipoxygenase enzyme system, and could be involved in the plant defense mechanism (Hildebrand, 1989). Alcohols, such as 1-hexanol, a relative of 2-ethyl 1-hexanol identified in this study, also have antifungal activity and prevent diseases (Archibold et al., 1997).

Bacterial volatiles also promote growth of plants (Ryu et al., 2003a). 2,3-Butadienol, enhanced the growth of *Arabidopsis thalliana* (Ryu et al., 2003a), and inhibited the pathogen *Erwinia carotovora* (Ryu et al., 2003b). Production of inhibitory volatiles may increase the survival rate of bacteria in soil, by eliminating potential competitors for nutrients (Mackie and Wheatley, 1999). When we observe a healthy crop and assume it to be devoid of pathogen inoculum, it may be due to the presence and activity of these volatile producers keeping populations of pathogens at low levels. Most of these bacteria have been isolated from soil, as root and rhizosphere colonizers. The role of these bacteria in stimulating the lipoxygenase pathway while colonizing the roots and, stimulating the production of antifungal plant volatiles, such as (*E*) 2-hexenal that could help in the control of soilborne pathogens as *S. sclerotiorum*, *Phytophthora* spp., *Pythium* spp., etc., needs to be investigated. Even though Ryu et al. (2003a) established the role of bacterial volatile 2,3-butadienol in disease suppression through induced resistance (IR), they failed to clearly establish if the disease suppression was due to IR or the antibacterial nature of the volatiles produced inside the sealed chamber. Our study is the first of its kind that has characterized and established the antifungal nature of organic volatiles produced by bacteria and their potential use in the biocontrol of plant pathogens. Studies are under way, in greenhouse and under field conditions, to look at the efficiency of bacteria to produce these antifungal volatiles in natural soil conditions, to reduce sclerotial viability of *S. sclerotiorum*, their continued existence and population dynamics, and their role in induction of the lipoxygenase pathway in canola and production of antifungal plant volatiles in biocontrol of *S. sclerotiorum*.

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References

Abawi, G.S., Grogan, R.G., 1979. Epidemiology of plant diseases caused by *Sclerotinia* species. Phytopathology 69, 899–904.

Altman, J., Lawlor, S., 1966. The effect of some chlorinated hydrocarbons on some soil bacteria. Journal of Applied Bacteriology 29, 260–265.

Andersen, R.A., Hamilton-Kemp, T.R., Hildebrand, D.F., McCraken Jr., C.T., Collins, R.W., Fleming, P.D., 1994. Structure–antifungal activity relationships among C_6 and C_9 aliphatic aldehydes, ketones and alcohols. Journal of Agricultural and Food Chemistry 42, 1563–1568.

Andrews, J.H., 1992. Biological control of the phyllosphere. Annual Review of Phytopathology 30, 603–605.

Archibold, D.D., Hamilton-Kemp, T.R., Barth, M.M., Langlois, B.E., 1997. Identifying natural volatile compounds that control gray mold (*Botrytis cinerea*) during postharvest storage of strawberry, blackberry and grape. Journal of Agricultural and Food Chemistry 45, 4032–4037.

DeMilo, A.B., Lee, C.J., Moreno, D.S., Martinez, A.J., 1996. Identification of volatiles derived from *Citrobacter freundii* fermentation of a trypticase soy broth. Journal of Agricultural and Food Chemistry 44, 607–612.

Domsch, K.H., 1959. Die wirkung von bodenfungiciden III. Quantitative varenderungen der bodenflora. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 66, 17–26.

Fernando, W.G.D., Linderman, R., 1994. Inhibition of *Phytophthora vignae* and root rot of cowpea by soil bacteria. Biological Agriculture and Horticulture 12, 1–14.

Fiddaman, P., Rossall, S., 1994. Effect of substrate on the production of antifungal volatiles from *Bacillus subtilis*. Journal of Applied Bacteriology 76, 395–405.

Gerlagh, M., Goosen Van de Geijn, H.M., Fokkemma, N.J., Vereijken, P.F.G., 1999. Long-term biosanitation by application of *Coniothyrium minitans* on *Sclerotinia sclerotiorum*-infected crops. Phytopathology 89, 141–147.

Gracia-Garza, J., Reedeler, R., Paulitz, T., 1997. Degradation of sclerotia of *Sclerotinia sclerotiorum* by fungus gnats (*Bradysia coprophila*) and the biocontrol fungi *Trichoderma* spp. Soil Biology & Biochemistry 29, 123–129.

Green-McDowelle, D., Ingber, M.B., Wright, M.S., Zeringue Jr., H.J., Bhatnagar, D., Cleveland, T.E., 1999. The effect of selected cotton-leaf volatiles on the growth, development and aflatoxin production of *Aspergillus parasiticus*. Toxicon 37, 883–893.

Gutterson, N., Ziegle, J.S., Warren, G.J., Layton, T.J., 1988. Genetic determinants for catabolite induction of antibiotic biosynthesis in *Pseudomonas fluorescens* HV 37a. Journal of Bacteriology 170, 380–385.

Handelsman, J., Stabb, E.V., 1996. Biocontrol of soilborne plant pathogens. The Plant Cell 8, 1855–1869.

Hildebrand, D.F., 1989. Lipoxygenases. Physiologia Plantarum 76, 249–253.

Howell, C.R., Stipanovic, R.D., 1979. Control of *Rhizoctonia solani* on cotton seedlings by *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology 69, 480–482.

Howell, C.R., Beier, R.C., Stipanovic, R.D., 1988. Production of ammonia by *Enterobacter cloacae* and its possible role in the biological control of Pythium preemergence damping-off by the bacterium. Phytopathology 78, 1075–1078.

Huang, H., Kokko, E., 1993. *Trichothecium roseum*, a mycoparasite of *Sclerotinia sclerotiorum*. Canadian Journal of Botany 71, 1631–1638.

Huang, H., Huang, J.W., Saidon, G., Erickson, R., 1997. Effect of allyl alcohol and fermented agricultural wastes on carpogenic germination of sclerotia of *Sclerotinia sclerotiorum* and colonization of *Trichoderma* spp. Canadian Journal of Plant Pathology 9, 43–46.

James, D.W., Gutterson, N.I., 1986. Multiple antibiotics produced by *Pseudomonas fluorescens* HV 37A and their differential regulation by glucose. Applied and Environmental Microbiology 52, 1183–1189.

Kanner, D., Gerber, N.N., Bartha, R., 1978. Pattern of phenazine pigment production by strain of *Pseudomonas aeruginosa*. Journal of Bacteriology 134, 690–692.

Kobaisy, M., Tellez, M.R., Webber, C.L., Dayan, F.E., Schrader, K.K., Wedge, D.E., 2001. Phytotoxic and fungitoxic activity of the essential oil of kenaf (*Hibiscus cannabinus* L.) leaves and its compositions. Journal of Agricultural and Food Chemistry 49, 3768–3771.

Loper, J.E., Buyer, J.S., 1991. Siderophores in microbial interactions on plant surfaces. Molecular Plant–Microbe Interactions 4, 5–13.

Mackie, A.E., Wheatley, R.E., 1999. Effects of the incidence of volatile organic compound interactions between soil bacterial and fungal isolates. Soil Biology & Biochemistry 31, 375–385.

Martens, J., Seaman. G., Atkinson. G (Eds.), 1994. Diseases of Field Crops in Canada. The Candadian Phytopathological Society Canada, 160 pp.

Oedjijono, M., Line, A., Dragar, C., 1993. Isolation of bacteria antagonistic to a range of plant pathogenic fungi. Soil Biology & Biochemistry 25, 247–250.

Robinson, P.M., McKee, N.D., Thompson, L.A.A., Harper, D.B., Hamilton, J.T.G., 1989. Autoinhibition of germination and growth in *Geotrichum candidum*. Mycological Research 93, 214–222.

Rothrock, C.S., Gottlieb, D., 1984. Role of antibiosis in antagonism of *Streptomyces hygroscopicus* var *geldanus* to *Rhizoctonia solani* in soil. Canadian Journal of Microbiology 30, 1440–1447.

Ryu, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Wei, H.X., Pare, P.W., Kloepper, J.W., 2003a. Bacterial volatiles promote growth in *Arabidopsis*. Proceedings of the National Academy of Sciences 100, 4927–4932.

Ryu, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Pare, P.W., Kloepper, J.W., 2003b. Volatiles produced by PGPR elicit plant growth promotion and induced resistance in Arabidopsis, Proceedings of the Sixth International Workshop on Plant Growth Promoting Rhizobacteria 2003 pp. 436–443.

Savchuk, S., 2002. Evaluation of biological control of *Sclerotinia sclerotiorum* on canola (*Brassica napus*) in the laboratory, in the greenhouse and in the field. MSc Thesis Dissertation, University of Manitoba.

Shojania, S., McComb, M.E., Oleschuk, R.D., Perreault, H., Gesser, H.D., Chow, A., 1999. Qualitative analysis of complex mixtures of VOCs using the inside needle capillary adsorption trap. Canadian Journal of Chemistry 77, 1716–1727.

Thaning, C., Welch, C.J., Borowicz, J.J., Hedman, R., Gerhardson, B., 2001. Supression of *Sclerotinia sclerotiorum* apothecial formation by the soil bacterium *Serratia plymuthica*: identification of a chlorinated macrolide as one of the causal agents. Soil Biology & Biochemistry 33, 1817–1826.

Thomashow, L.S., Weller, D.M., 1988. Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var *tritici*. Journal of Bacteriology 170, 3499–3508.

Tu, J., 1985. Tolerance of white bean (*Phaseolus vulgaris*) to white mold (*Sclerotinia sclerotiorum*) associated with tolerance to oxalic acid. Physiological Plant Pathology 26, 111–118.

Vincent, M.N., Harrison, L.A., Brackin, J.M., Kovacevich, P.A., Mukerji, P., Weller, D.M., Pierson, E.A., 1991. Genetic analysis of the antifungal activity of a soilborne *Pseudomonas aureofaciens* strain. Applied and Environmental Microbiology 57, 2928–2934.

Voisard, C., Keel, D., Defago, G., 1989. Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. European Molecular Biology Organization Journal 8, 351–358.

Yuen, G.Y., Godoy, G., Steadman, J.R., Kerr, E.D., Craig, M.L., 1991. Epiphytic colonization of dry edible bean by bacteria antagonistic to *Sclerotinia sclerotiorum* and potential for biological control of white mold disease. Biological Control 1, 293–301.