Use of bacterial organic volatiles in biocontrol of *Sclerotinia sclerotiorum* in canola

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ABSTRACT

Sclerotinia sclerotiorum is a pathogen on many important field crops grown in Canada including canola (*Brassica napus*). We have identified soil and root bacteria that are capable of producing organic volatile compounds that inhibit sclerotia germination, and mycelial growth of the Sclerotinia pathogen *in vitro* (divided culture plates) and *in vivo* (soil). Ascospore germination, done in cavity slides, was inhibited 54-90% by volatile producers. When mycelial plugs or sclerotia exposed to these volatiles were transferred to fresh agar plates, the pathogen could not grow, indicating that volatiles were fungi-toxic to Sclerotinia propagules. Head space volatiles produced by bacteria were collected with activated charcoal traps by passing nitrogen over 48 h continuous shake cultures, eluted with methylene chloride and identified using GC-MS analysis. The volatile compounds identified were alcohols, ketones, sulfides, or aldehydes. Of assays carried out in divided Petri plates with filter-disks soaked with these compounds (100-150 µl) only six compounds completely inhibited mycelial growth or sclerotia formation, signifying their role in biological control. Volatiles may be important in disease management on residue in soil. Studies are underway to understand this phenomenon.

Key Words: Biological control – Sclerotinia – bacteria – volatiles - canola

INTRODUCTION

Canola (*Brassica napus* L.) is one of the largest crops grown in Manitoba. Approximately 66 per cent of the country's edible oil industry depends on canola. Annual export of canola products from Canada is around $ 2 billion.

Stem rot caused by *Sclerotinia sclerotiorum* is an economically important disease of canola. The fungus is soil borne but infection occurs at flowering stage by wind borne ascospores. The challenge to control *Sclerotinia* stem rot of canola has been made more difficult with the phasing out of Benlate (Benomyl). Rotations have not been very effective, as the pathogen has a wide host range and attacks several crops grown in rotation, and as the spores could travel long distance and cause infection in neighboring fields. The overwintering sclerotia can survive for long periods, and there is no commercial cultivar that is resistant to the pathogen.

Biological control is an effective alternative. We have developed a bacterial spray application that targets the flowering stage where the bacterium or its products can inhibit ascospore germination (Savchuk *et al.,* 2000). The objective of the present study was to search for volatile antibiotics of bacterial origin to control the sclerotial phase in the soil.

MATERIALS AND METHODS

The pathogen *S. sclerotiorum* strain SS33, and 13 bacteria identified as volatile organic compound producers were used in the experiment. They are: isolates 29; 33; 35 (*Pseudomonas fluorescens* (Bio Type-G); 41 (*Pseudomonas* spp.); BCA$^-$ (*P. chlororaphis)* (BT-D);, 190 (*P. chlororaphis* (BT-D); 191; 199; 200 (*P. aurantiaca*); 202 (*P. chlororaphis* (BT-D); 209 (*P. chlororaphis* (BT-D); 210 (*P. chlororaphis* (BT-D); and strain 223.

Production of volatile antibiotics: Divided plate method

A divided Petri plate (85 mm dia) was used. One half of the plate containing potato dextrose agar (PDA) was inoculated with a 5 mm mycelial disc of the pathogen at the periphery. In the second half of the plate, the bacterial isolate was streaked on to tryptic soy agar (TSA) media. The plates were sealed tightly after inoculation and further incubated at 25^{o}C for 7 days. The growth of the fungus was measured compared to the control. After 7 days the mycelial plugs from all the plates were removed and tested for viability in a fresh Petri plate containing PDA.

Sclerotial inhibition

The above procedure was used with partial modification. Surface sterilized sclerotia were placed on the side of PDA. Bacteria were streaked on TSA side. After 7 days, the sclerotium was removed and tested for its viability in a fresh plate containing PDA medium.

Ascospore inhibition

A known dilution of ascospores ($5x\ 10^{4}$) in phosphate buffer (pH 7.0) was placed in a cavity slide. The slide was then kept inside the bottom lid of a sterile Petri plate. The top lid contained the bacterial culture. The plates 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. The percentage inhibition was calculated.

Production of volatile antibiotics in the soil substrate/pot mixture

Petri plates (150 mm dia) were used. The bottom lid of the plate was filled with sterilized field soil or vermiculite to a depth of one cm. Ten uniform (size) sclerotia packed inside a netted nylon pouch were placed at the bottom of the plate. In a second Petri plate bacterial isolate was streaked in TSA medium. The TSA plate was placed as the top lid over the first plate containing the sclerotia and tightly sealed so that volatile organic compounds did not escape. After 10 days of incubation, the buried sclerotia were collected and tested for their survival in fresh medium.

Collection of volatile organic compounds

Methods by DeMilo *et al.,*(1996) was followed with little modification. The bacterial isolates were shake-cultured in TS broth in 250 ml conical flasks fitted with two-way rubber cork having glass tubes inserted through them. The neck was tightly sealed with Para film to prevent the escape of volatile organic compounds. One of the inserted glass tubes was placed just one cm above the bacterial culture. The other end of the tube was connected to a nitrogen supply system for removing the headspace volatiles. The second tube was connected to a trap containing activated charcoal (300 mg; 20 to 40 mesh). After removing the volatiles for 10 h from the headspace, the trap was physically removed and eluted with 0.5 ml of methylene chloride.

GC-MS analysis

Gas chromatography was performed using a Varian Star 3400 CX series GC with a flame ionization detector. The nature of volatiles was determined from a qualitative analysis of the total ion chromatograms from each GC/MS run through a personal computer.

In-Vitro analysis of antifungal organic volatile compounds and identification of bacteria

The GC/MS analysis revealed the presence of alcohols, aldehydes, ketones, esters, long chained aliphatic and aromatic organic compounds. Each of the compounds was tested for antifungal activity by placing 100µl and 150µl of each compound, singly and in combination with tryptic soy broth (TSB), on sterile filter paper discs placed in divided agar plates.

All bacteria were identified to the species level, using the Microlog^{TM} (Biolog Inc., Hayward, California, USA) system available in our laboratory.

RESULTS

When volatile producing bacteria were screened against *Sclerotinia* on divided plates, strains BCA^- , 33, 41, 190, 191, 199, 200, 202, 209, 210, 220 and 223 completely inhibited growth of the fungal mycelia, and sclerotia germination. Strain 35 was able to significantly reduce sclerotia germination, but not mycelial growth. When mycelial plugs or sclerotia were transferred to new PDA plates, only those from the control plates grew, suggesting fungi-toxic (fungicidal) effects from volatile antibiotics. However, when buried sclerotia were placed on fresh PDA plates, only strains 200 and 209 significantly reduced germination and growth. Ascospore germination in glass slides was inhibited by 54-90% in the presence of the volatile producing strains of bacteria. There were specific volatiles produced by some bacteria, and several which were common to many strains (Table 1). Several Pyrazine, and benzalddehyde compounds were isolated, but were present in the plain tryptic soy broth (TSB) as well, indicating they were to be independent of bacterial action. Of the diverse compounds tested, only six compounds had 100% inhibition of the fungal mycelium. The combination with TSB did not influence the mycelial inhibition of any of the compounds tested. The six compounds that had 100% inhibition of the fungal mycelium, in both the amounts tested, were 2-ethyl-1-hexanol, cyclohexanol, decanal, nonanal, dimethyl trisulfide and benzothiazole (Table 1).

Table 1: Organic Volatile Compounds identified to give 100% inhibition of *S. sclerotiorum* growth

Name of Compound	Bacterial strain it was isolated from
Nonanal	202, 210, 220, BCA^-
Cyclohexanol	210
Benzothiazole	220, BCA^-
2-ethyl, 1-hexanol	29, 33, 35, 41, 191, 199, 202, 210, 220, 223, BCA^-
N-Decanal	202, 220
Dimethyl trisulfide	199, 202
Benzaldehyde	TSB medium, several bacterial cultures
Pyrazine derivatives	TSB medium, several bacterial cultures

DISCUSSION

Bacteria isolated from canola root and stubble, and from soybean roots produced volatiles that were inhibitory to survival, infection and reproductive structures of *Sclerotinia sclerotiorum*. Interestingly, none of the phylloplane bacteria isolated from leaves and flowers of canola had any volatile production. This is an interesting phenomenon; if volatiles are to be effective in controlling the pathogen with the production of volatile antibiotics, the most effective place would be in soil as these volatiles will come in direct contact with sclerotinia propagules. In another study we identified oxalate oxidase producing bacteria, and they were from the phylloplane or flowers. If oxalic acid (pathogenicity factor) 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 bacterial species (*Pseudomonas* sp.) are common rhizosphere bacteria, while the oxalate oxidase producers, *Pantoea agglomerans* are common epiphytic phyllosphere bacteria.

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