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Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary by *Pseudomonas* and *Bacillus* species on canola petals

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Abstract

Four bacterial strains, *Pseudomonas chlororaphis* (PA-23), *Bacillus amyloliquefaciens* (BS6), *Pseudomonas* sp. (DF41) and *B. amyloliquefaciens* (E16) which had been found to have biocontrol activity in vitro assays against *Sclerotinia sclerotiorum*, the causal agent of stem rot of canola, were tested for their efficacy in greenhouse and field conditions.

Microscopic studies showed that *P. chlororaphis* strain PA-23 inhibited the germination of *S. sclerotiorum* ascospores on petals while complete colonization of petals was observed 48 h after application of ascospores alone. Double application of PA-23 on canola plants challenged with the ascospores of *S. sclerotiorum* triggered increased levels of hydrolytic enzymes including chitinase and β-1,3-glucanase in canola plants. In addition, it also triggered the expression of the pathogenesis-related protein PR3.

Field studies over a period of two years indicated that disease control with PA-23 and BS6 was comparable to that achieved with the fungicide Rovral Flo® (iprodione). There was no significant difference between single- and double-spray application of PA-23 and BS6 in the management of canola stem rot. Results suggest that *P. cholororaphis* PA-23 and *B. amyloliquefaciens* BS6 can be used to control Sclerotinia stem rot of canola under field conditions.

Keywords: *Sclerotinia sclerotiorum*; Biocontrol; Canola; *Pseudomonas* sp.; *Bacillus* sp.; Petals; Phyllosphere

1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is the causal agent of disease on more than 400 plant species, including canola, on which it causes stem rot (Nelson, 1998). It is one of the most important diseases of canola in Western Canada (Martens et al., 1994), causing yield losses of 5–100% (Manitoba Agriculture, 2002). It is difficult to breed for resistance to *S. sclerotiorum* since resistance is governed by multiple genes (Fuller et al., 1984). Control using crop rotations is unrealistic due to the persistence of survival structures (sclerotia) in the soil for long periods and because Sclerotinia has such a wide host range (Nelson, 1998). These factors necessitate the use of fungicides, which have been known to have adverse effects on non-target organisms (Gilmour, 2001; McGrath, 2001; Rose, 1995). The USA has recently put a dry bean consignment from Canada under quarantine and then rejected it as it was found to be contaminated with 0.021 ug g^{-1} vinclozolin (Ronilin®) residue on the edible part, which exceeds the 0.0 ug g^{-1} acceptance limit in USA (Mark Goodwin, personal communication). Therefore, the relatively unreliable control with traditional methods and concerns about pesticide residues has prompted interest in biological control as an alternative strategy for disease management.

In addition, as most inoculum is ascosporic, a few germinating sclerotia can lead to significant infection levels in the field (Davies, 1986). Also, ascospores could travel long distance from neighbouring fields and infect petals (Venette, 1998). Therefore, there is a need for research into biocontrol of *S. sclerotiorum* on canola, specifically, on limiting petal infection by ascospores. Foliar applications

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of biocontrol agents are important in the Sclerotinia-canola system, as the ascospores generally infect senescing petals at the flowering stage (Turkington and Morrall, 1993). Related studies have demonstrated that younger rapeseed flowers, which were inoculated with *S. sclerotiorum* ascospores, showed more disease on rapeseed compared to older flowers (Inglis and Boland, 1990). As this is a narrow window to protect the plant from infection, biological control may work well in controlling the germination of ascospores on petal surfaces.

Studies on phyllosphere biological control of Sclerotinia on canola with bacterial antagonists have not been reported previously. *Erwinia* spp. and *Bacillus* spp. have been shown to inhibit *Sclerotinia* in vitro and in vivo and control Sclerotina rot on bean (Godoy et al., 1990; Huang et al., 1993; Yuen et al., 1991, 1994; Tu, 1997). Inconsistent results were found using *Erwinia herbicola* and *Bacillus subtilis* to control white mold of bean in fields (Yuen et al., 1992; Boland, 1997). Populations of *E. herbicola* declined from 10^7 cfu per blossom to 10^5 cfu per blossom within 8 days after application (Yuen et al., 1991). The rate of decline was faster in blossoms than leaves, which was due to the reduced nutritional status of blossoms (Yuen et al., 1994). Density of *P. chlororaphis* at 10^3 cfu g^{-1} of fresh roots promoted plant growth of pepper and suppressed *P. aphanidermatum* (Khan et al., 2003).

Plants have various defense mechanisms to counteract invading pests and pathogens. Induce systemic resistance (ISR) is activated by plant growth promoting rhizobacteria, (PGPR) rendering spatially distant, plant parts with resistance to pathogens. ISR mediated through bacterial antagonists has been well documented in bean, carnation, cucumber, radish, tomato, and *Arabidopsis thaliana* and they confer resistance to broad spectrum of pathogens (Van loon et al., 1998). ISR is associated with accumulation of PR proteins (Viswanathan and Samiyappan, 1999, 2001) and synthesis of phenolics (M'Piga et al.1997; Chen et al., 2000). PR proteins include defense-related lytic enzymes such as chitinase (PR-3) and β-1,3-glucanase (PR-2) which hydrolyse the chitin and β-1,3-glucan, major components of fungal pathogen cell wall. Colonization of cucumber plants and bean roots with *Pseudomonas corrugata* increased the levels of phenylalanine ammonia lyase (PAL) (Chen et al., 2000). Bean root colonization with PGPR triggered the accumulation of mRNAs encoding PAL and chalcone synthase (Zdor and Anderson, 1992). Apart from the increase of PAL, colonization of bean rhizosphere by *P. aeruginosa* 7NSK2 increased the salicylic acid levels in leaves and activated the systemic resistance pathway (De Meyer et al., 1999). In addition to PAL, rhizosphere colonization by PGPR in bean (Zdor and Anderson, 1992) and cucumber (Chen et al., 2000) induced peroxidase (PO) activity, which aids in the synthesis of lignin and oxidative phenols, thereby conferring resistance to plant pathogens. PGPR-mediated resistance is activated through the jasmonic acid and ethylene pathways.

Preliminary studies in vitro identified *P. chlororaphis* PA-23 and *Pseudomonas* sp. DF41 to be very effective inhibitors of *S. sclerotiorum* mycelial growth and germination of sclerotia and ascospores (Savchuk, 2002). The two bacteria also have significant ($P > 0.05$) disease suppression in the greenhouse (Savchuk and Fernando, 2004; Savchuk, 2002). Greenhouse studies have also shown that *B. amyloliquefaciens* (BS6), and *B. amyloliquefaciens* (E16) are effective biocontrol agents against *Sclerotinia* (Zhang, 2004; Ramarathnam and Fernando, 2003). Therefore the objectives of this study were (i) to investigate the nature of inhibition of ascospores on petals in the presence of biocontrol bacteria PA-23, (ii) to study the induction of lytic enzymes by PA-23 in biocontrol of Sclerotinia and (iii) to investigate the bacterial biocontrol of Sclerotinia in canola in the field.

2. Materials and methods

2.1. Bacterial isolates and S. sclerotiorum inoculum production

Bacterial strains, *P. chlororaphis* (PA-23), *Pseudomonas* sp. (DF41), *B. amyloliquefaciens* (BS6), and *B. amyloliquefaciens* (E16) were used to assess their antagonistic potential against *S. sclerotiorum* under field conditions. Strain PA-23 was also studied for the triggering of induced resistance in canola plants and for the inhibition of ascospores on canola petals. The sources and identifications of bacteria used in this study are listed in Table 1. Rifampicin-resistant (at 150 mg l^{-1}) strains of all isolates were developed (Zhang, 2004). The bacterial strains that had been stored in Nutrient Agar (Difco Laboratories, Detroit, MI, USA) broth with 20% glycerol (−80 °C) were streaked onto Luria Bertani Agar plates [LBA contains

Table 1
Bacterial sources and identification

Strain	Source	Identification	References
PA-23	Soybean, root tip	*Pseudomonas chlororaphis*	Savchuk (2002)
DF41	Canola, root tip	*Pseudomonas* spp.	Savchuk and Fernando (2004)
BS6	Canola, leaf	*Bacillus amyloliquefaciens*	Zhang (2004)
E16	Canola, leaf endophyte	*Bacillus amyloliquefaciens*	Ramarathnam and Fernando (2003)

Note: Bacterial identifications are based on the Biolog Microplates™ (Microlog, Hayward, CA, USA) and accompanying software.

15.0 g agar technical, 10.0 g tryptone peptone, 5.0 g yeast extract (Difco Laboratories, Detroit, MI, USA), 5.0 g NaCl l^{-1}]. A single colony of each strain was inoculated and grown in LB broth with constant shaking at 150 rpm for 48 h at room temperature. The cultures were centrifuged at 6000 rpm for 10 min and bacterial cells were resuspended in phosphate buffer (100 mM, pH. 7.0). The cell concentration was adjusted spectrophotometrically to 9×10^8 cfu ml^{-1} (0.3 OD at 595 nm). The ascospores of *S. sclerotiorum* were purchased from Dr. M.G. Boosalis, Department of Plant Pathology, University of Nebraska, Lincoln, NE, USA.

2.2. Antifungal efficacy of P. chlororaphis (PA-23) on ascospore germination

PA-23 performed best for inhibition of germination of ascospores of *S. sclerotiorum* and was investigated further for antifungal activity. Canola plants were sprayed twice at 30% and 50% bloom with PA-23 (9×10^8 cfu ml^{-1}) amended with 0.02% Tween 20® until run off. The plants were kept in a high humidity chamber (23/16 °C, 16 h photoperiod) for 24 h. After 24 h, canola petals were sprayed with ascospores of *S. sclerotiorum* (8×10^4 ml^{-1}) suspended in 0.1 M phosphate buffer. Twenty-five petals were sampled randomly from the treated plants at 9, 18, 24 and 48 h after application of ascospores. Percent germination of ascospores was observed microscopically after processing of petals.

Petals were processed by transferring them to the surface of filter paper in glass Petri plates. The filter paper was placed on absorbent cotton pads soaked in 1:1 mixture (v/v) of 95% ethanol and glacial acetic acid (Fischer Scientific, Nepean, ON). Plates were sealed with parafilm and incubated at room temperature for 48 h, until pigments were cleared from the petals. Cleared petals were then placed onto 25 × 75 mm microscopic slides and stained with a drop of 0.01% cotton blue in lactoglycerine (1:2:1 water: glycerol: lactic acid). The number of germinated ascospores was observed with an epifluorescence microscope (Zeiss, Germany) at 400 × magnification and microscopic fields were also photographed. Twenty-five petals were investigated for each treatment. The Duncan's multiple range test (DMRT) was used to test for variation in percent germination of ascospores between treatments at different time intervals.

2.3. Sample collection and enzyme extraction

Chitinase and β-1,3-glucanase are the key enzymes involved in the lysis of fungal cell walls. The effect of PA-23 on the induction of hydrolytic enzymes chitinase and β-1,3-glucanase was investigated under greenhouse conditions. Treatments are as follows: Treatment 1: Canola plants were sprayed twice at 30% and 50% bloom with PA-23 (9×10^8 cfu/ml). The time between 30% and 50% bloom was 24 h. 24 h after the second spray of PA-23, the canola plants were sprayed with ascospores of *S. sclerotiorum* (8×10^4 ml^{-1}) suspended in 0.1 M phosphate buffer; Treatment 2: Canola plants were sprayed at 30% and 50% with PA-23 (9×10^8 cfu ml^{-1}); Treatment 3: Canola plants were sprayed with distilled water alone (healthy control); Treatment 4: Canola plants were sprayed with ascospores of *S. sclerotiorum* (8×10^4 ml^{-1}) suspended in 0.1 M phosphate buffer (Inoculated control). Tween® 20 (0.02%) was used as a surfactant in all inoculations. Treated plants were transferred to a high humidity chamber (23/16 °C, 16 h photoperiod) and kept for 10 days. Inoculated petals fell on leaf surfaces of the canola plants. Parts of the leaf surrounding the fallen canola petals were collected at 1, 2, 4, 6 and 8 days after ascospore spray for enzyme assay.

2.4. Assay for chitinase detection

One gram of the fresh leaves were homogenized with liquid nitrogen immediately after sampling and the powdered sample was extracted with 5 ml of 0.1 M sodium citrate buffer, pH 5.0 for 1 min at 4 °C. The homogenate was centrifuged for 10 min at 10,000*g* (Sorvall Super T 21; Dupont, Wilmington, NC, USA) at 4 °C. The supernatant was used for spectrophotometric estimation of chitinase, and the enzyme activity was expressed as nmol *N*-acetyl-glucosamine (GlcNAc) equivalents per min. per g of leaf or rhizome (Boller and Mauch, 1988).

2.5. Assay for β-1,3-glucanase detection

One gram of the fresh leaves was homogenized in 1 ml of 0.1 M phosphate buffer immediately after sampling. The homogenate was centrifuged at 15,000*g* for 15 min at 4 °C and the supernatant was used as the enzyme source. Crude enzyme extract of 62.5 µl was added to 62.5 µl of 4% laminarin and incubated at 40 °C for 10 min. The reaction was stopped by adding 375 µl of dinitro salicylic acid and heated for 5 min in a boiling water bath. The resulting solution was diluted with 4.5 ml distilled water and the absorbance was read at 500 nm. The crude extract preparation with laminarin with zero time incubation served as blank. The enzyme activity was expressed as µg equivalent of glucose per min per gram fresh weight (Pan et al., 1991).

2.6. Western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12% polyacrylamide gels (Sigma, USA). A medium range protein marker (Sigma, USA) was used as a molecular mass standard. After electrophoresis, proteins were electrotransfered from the gel to a nitrocellulose membrane (Sigma, St Louis, MO, USA). The tobacco chitinase antiserum (from Dr. Legrends lab, Strasbourg, France) was used to

detect chitinase. The bands were visualized in an alkaline phosphatase reagent containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma) as described by Gallagher et al. (1995).

2.7. Field trial

Biocontrol of *S. sclerotiorum* by bacterial strains DF41, PA-23, E16 and BS6 were evaluated under field conditions. Two field trials were conducted during summers of 2003 and 2004. Both trials were conducted at the University of Manitoba Carman Research Station in Carman, Manitoba, Canada. The block was situated on a well-drained, clay loam soil (Denham clay loam). The plots were treated with Roundup Transorb™ (480 g l^{-1} glyphosate, Monsanto, Missisauga, Ont., Canada) at a rate of 0.82 l ha^{-1}, and DECIS® (27.5 g l^{-1} deltamethrin, 811 g l^{-1} liquid hydrocarbons, Bayer Crop Science) at a rate of 0.74 l ha^{-1}. Plots were seeded with Roundup Ready canola (LG3235) seeds pre-treated with HELIX, which contains insecticides (thiamethoxam) and the fungicides (difenoconazole, metalaxyl-*M* and fludioxonil) at the rate of 10 kg ha^{-1}. Recommended dosage of fertilizer (23-24-0) was applied while seeding (80 kg ha^{-1}).

The following treatments were included in the experiment: (1) ascospore inoculation (control); (2) fungicide spray (Rovral Flo® 240 g l^{-1} iprodione, Bayer Crop Science, Calgary, Alberta, Canada) at the recommended rate of 2.10 l ha^{-1}, (3) DF41 (single spray application); (4) PA-23 (single spray application), (5) PA-23 (two spray applications), (6) E16 (single spray application), (7) BS6 (single spray application) and (8) BS6 (two spray applications). The experimental design was a randomized complete block with four replications. Individual plots were 4×4 m. Plots were sprayed at 30% bloom with the bacterial strains (9×10^8 cfu ml^{-1}) or fungicide treatments. The second bacterial spray was applied at 50% bloom. Ascospores were sprayed at a concentration of 8×10^4 ml^{-1}. All plots were sprayed with ascospores at 50% bloom, with a uniform 4.0 l of spray volume per plot. Inoculated control plots received ascospore sprays alone. Petal infestation by the ascospores of *S. sclerotiorum*, and percent disease incidence (PDI) were recorded. The experiment was repeated in June 2004.

Petal infestation by *S. sclerotiorum* in the field was investigated in both trials. Each treatment was sampled 3 days after ascospore inoculation. Twenty petals per plot were sampled and placed on PDA media amended with streptomycin sulphate (200 mg l^{-1}, Sigma®, St. Louis, MO, USA) with four petals per plate. Colonies of *S. sclerotiorum* were identified visually within 5 days and confirmed through sub-culturing.

Percent disease incidence on stem was assessed at maturity in both trials. Sixty plants per plot in a 1×1 m quadrat were scored for the presence or absence of stem rot. Data was subjected to analysis of variance using the Statistical Analysis System (SAS Institute, Cary, NC, USA) followed by mean separation by LSD ($P \leqslant 0.05$) (Sall and Lehman, 1996).

3. Results

3.1. Antifungal action of P. chlororaphis (PA-23) against ascospore germination

Ascospore germination was initiated 9 h after the ascospore spray. Eighteen hours later there was considerable branching of germ tubes, followed 24–48 h later by colonization of petals in the ascospores only treatment (Fig. 1E–H). However, on the petals that received an application of PA-23, very few ascospores initiated the germination after 24 and 48 h of ascospore spray, which seems to explain the antifungal action of PA-23 in inhibiting the germination of ascospores (Fig. 1A–D).

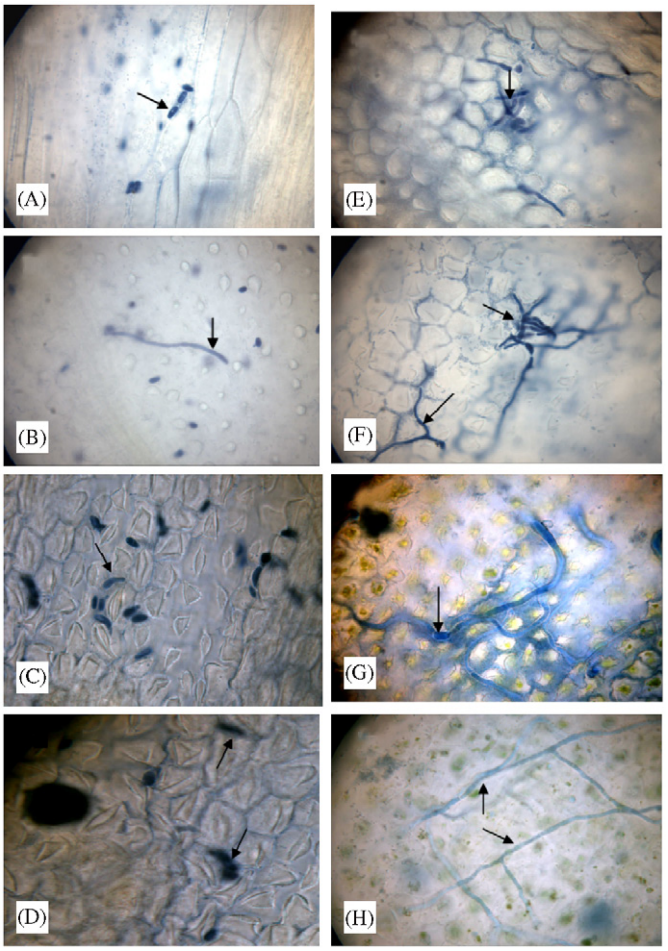

Fig. 1. Microscopic examination of ascospore germination of *S. sclerotiorum* on canola petals in the presence or absence of *P. chlororaphis* (PA-23). (A–D) PA-23 + sclerotinia ascospores: isolate PA-23 sprayed on to petals at 10^8 ml^{-1} 24 h prior to inoculation with ascospores. (E–H) sclerotinia ascospores only: ascospores of *S. sclerotiorum* sprayed on to petals at 10^4 ml^{-1} without strain PA23. A–D and E–H refers to ascospore germination at 9, 18, 24 and 48 h, respectively. A–D and E–H are photographed at 400 magnification.

3.2. Induced systemic resistance

The results are the averages of the two repetitions. In order to determine whether data variances between two repetitions were homogeneous, Levene's test was employed. The test was not significant at $P<0.05$, indicating that variances were homogeneous across repetitions and therefore data were combined across two trials.

An application of PA-23 at both 30% and 50% bloom followed by challenge inoculation with *Sclerotinia* ascospores induced significantly higher chitinase and β-1,3-glucanse activity (Figs. 2 and 3). In contrast, the activity was less in the healthy control, ascospore inoculated control and PA-23 treatments. Accumulation of these hydrolytic enzymes reached the highest level 4 DAI for chitinase (2.5 fold increase over inoculated control) and 6 DAI for β-1,3-glucanase (3 fold increase over inoculated control), following with a slow decline there after.

One chitinase isoform with a molecular weight of 34 kDa was detected by western blotting using tobacco chitinase antiserum in PA-23 treated plants with or without challenge inoculation by *S. sclerotiorum*. The expression was very low in the inoculated control (Fig. 4).

3.3. Field evaluation of bacterial strains

Results from two field trials conducted during 2003 and 2004 indicate that either one or two applications of bacterial strains suppressed stem rot under field conditions (Table 2). Applications of PA-23 and BS6 twice at 30% and 50% bloom significantly reduced the percent canola stem rot incidence in both trials. However there was no significant difference between one and two sprays of BS6 and PA-23. Percent stem rot incidence with double application of PA-23 was 7.50% and 28.73%, and with double application of BS6 was 5.00% and 29.55% in trial I and II, respectively. In 2004, there was more infection in the field with the pathogen-inoculated control treatment having 20% and 44%. The results were comparable to the

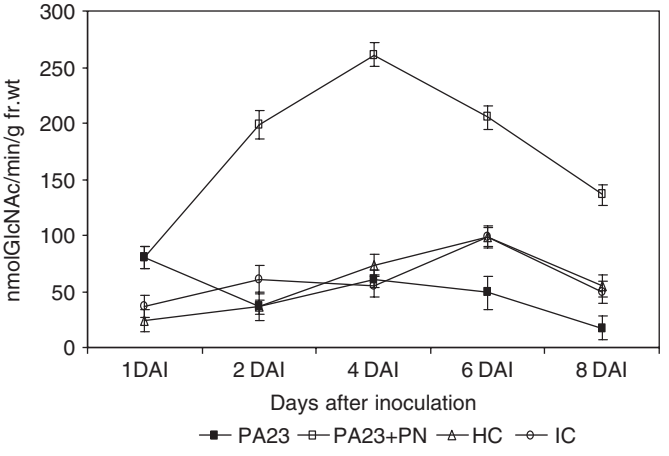

Fig. 2. Effect of spray application of *Pseudomonas chlororaphis* (PA-23) on the activity of chitinase with or without *S. sclerotiorum*. Chitinase activity was assayed after inoculation with the ascospores of *S. sclerotiorum* on 1, 2, 4, 6 and 8 days. DAI—days after inoculation; PA-23—*P. chlororaphis* (PA-23); PA-23 + PN—*P. chlororaphis* PA-23 challenged with ascospores of *S. sclerotiorum*; HC—plants sprayed with sterile distilled water—healthy control; IC—plants sprayed with ascospores of *S. sclerotiorum*—inoculated control.

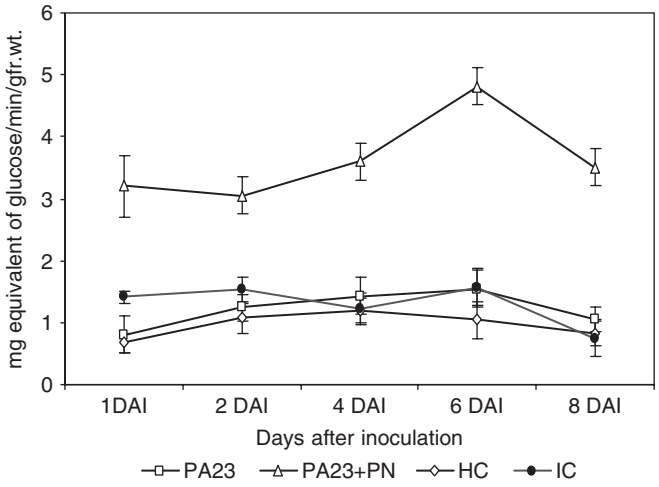

Fig. 3. Effect of spray application of *P. chlororaphis* (PA-23) on the activity of β-1,3-glucanase with or without *S. sclerotiorum*. Glucanase activity was assayed after inoculation with the ascospores of *S. sclerotiorum* on 1, 2, 4, 6 and 8 days after. DAI—days after inoculation; PA-23—*P. chlororaphis* (PA-23); PA-23 + PN—*P. chlororaphis* (PA-23) challenged with ascospores of *S. sclerotiorum*; HC—plants sprayed with sterile distilled water—healthy control; IC—plants sprayed with ascospores of *S. sclerotiorum*—inoculated control.

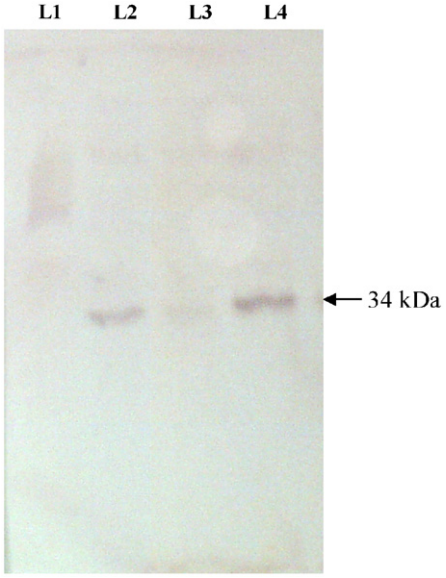

Fig. 4. Western blot analysis of chitinase in canola induced by *P. chlororaphis* (PA-23) with or without *S. sclerotiorum*. Lane 1—healthy; Lane 2—*P. chlororaphis* (PA-23) treated plants; Lane 3—healthy plants sprayed with ascospores of *S. sclerotiorum*; Lane 4—*P. chlororaphis* PA-23 treated plants inoculated with ascospores of *S. sclerotiorum*.

Table 2
Effects of bacterial biocontrol agents on Sclerotinia-ascospore petal infestation and stem rot incidence on canola (cv. Westar) under field conditions

Treatment	Trial I 2003		Trial II 2004	
	Percent petal infestation	PDI	Percent petal infestation	PDI
Pathogen-inoculated control	20.0a	33.8a	75.0a	44.1a
One application of Rovral Flo	7.5ab	7.5bc	66.7ab	35.8ab
One application of DF41	2.5b	23.8ab	71.7a	37.5ab
One application of PA-23	15.0ab	18.8abc	55.0c	27.9ab
Two applications of PA-23	5.0b	7.5bc	41.7d	28.7b
One application of E16	5.0b	22.5abc	68.3ab	36.9ab
One application of BS6	5.0b	13.8bc	60.0bc	35.4ab
Two applications of BS6	7.5ab	5.0c	38.3d	29.6b

Single spray applications with bacterial strains (9×10^8 cfu ml^{-1}) were done at 30% bloom. Double applications were carried out at 30% and 50% bloom. Sclerotinia inoculations were followed with an ascospore spray (8×10^4 ml^{-1}).
PDI—Percent disease incidence (stem rot infection); percentage data were arcsine transformed prior to analysis.
Values are the mean of three replications. Means followed by a common letter within a column are not significantly different ($P > 0.05$) according to Duncan's multiple range test (DMRT).

application of fungicide Rovral-Flo and were significantly different from pathogen-inoculated control. In both trials, petal infestation levels by *S. sclerotiorum* were lower in the plots that received two spray applications of PA-23 and BS6 than in those with one application. Differences between one and two sprays of either BS6 or PA-23 were not statistically different in trial I. In contrast, significant ($P \leqslant 0.05$) differences in petal infestation between one and two applications were observed in trial II.

4. Discussion

P. chlororaphis (strain PA-23) and *B. amyloliquefaciens* (strain BS6) significantly reduced stem rot caused by *S. sclerotiorum* under field conditions. Ascospore germination was inhibited and plant defence enzymes were triggered by *P. chlororaphis* (PA-23).

Increased concern over the impact of chemical pesticides on the environment has resulted in the increased interest in biocontrol strategies for the management of *S. sclerotiorum*. The most predominantly studied biocontrol agents for the management of *S. sclerotiorum* include mycoparasitic fungi and hypovirulent strains of the target fungus. The parasitic fungus *Coniothyrium minitans* has been widely studied as a biocontrol agent for *S. sclerotiorum*. Soil application of *C. minitans* reduces carpogenic germination of *S. sclerotiorum*, and it is commercially available as Contans® (Vrije et al., 2001). However, few attempts have been made to explore the possibility of bacterial biocontrol agents for the management of canola stem rot.

MASPEC analysis of the extracellular secretion from PA-23 revealed that it produced phenazine-1-carboxylic acid (PCA) and acetamido anthranilic phenol (AAP) (data not shown). *P. fluorescens* 2-79 suppresses take-all of wheat and has been shown to produce PCA and AAP (Slininger et al., 2000). Presence of phenazine biosynthetic genes and PCA production by PA-23 (Zhang et al., 2006) account for the inhibition of mycelial growth of *S. sclerotiorum* in vitro (Zhang and Fernando, 2004a, b). Primers were used with strain BS6 DNA to amplify the antibiotic genes encoding for Zwittermicin A, a relatively common antibiotic in *Bacillus sp*. Although, Zwittermicin A, a self-resistant gene was not identified (Zhang et al., 2006), further work has shown that it has biosynthetic genes for Bacillomycin D (Ramarathnam and Fernando, unpublished data). MASPEC analysis of the extracellular metabolites from BS6 indicated the presence of triacetin (data not shown). Triacetin also known as glyceryl triacetate, functions as a cosmetic biocide, and the presence of 1,2-glyceryl diesters in triacetin affect cell growth and proliferation (Fiume and Cosmetic Ingedient Review Expert Panel, 2003). Cosmetic biocides are antibiotics produced by microorganisms and used as an ingredient in cosmetics for humans, in foot creams and in facial products.

The successful use of biocontrol relies on an effective delivery system and subsequent survival of bacteria in the infection court. Since the primary infection of *S. sclerotiorum* on canola occurs through ascosporic infection of senescing petals (Adams and Ayers, 1979), the bacterial strains were delivered to petals as one or two spray applications during the blooming stage. Application of PA-23 at log 4 and log 8 cfu ml^{-1} was effective in inhibiting the ascospore germination of *S. sclerotiorum* on canola petals and suppressed the disease severity in greenhouse conditions (Savchuk and Fernando, 2004). Application of *B. subtilis* to blossoms reduced bean white mold over a 2-year field trial (Tu, 1997). However, Boland (1997) reported that *B. subtilis* did not consistently control white mold of bean under field conditions. We found that double applications of PA-23 and BS6 during 30% and 50% bloom were effective over the 2 years tested.

Stem rot severity on canola was highest when the ascospores were present prior to introduction of antagonistic bacteria onto petals (Savchuk and Fernando, 2004). Co-inoculation or pre-application of antagonistic bacterial strains suppressed the disease severity of *S. sclerotiorum*

(Savchuk and Fernando, 2004). Earlier work on delivery systems is in agreement with our work. Colonization of bean leaves by *C. minitans* was effective in preventing *Sclerotinia* ascospore infection on beans (Bardin and Huang, 2001). Pre-treatment of bean plants during bloom with *Erwinia herbicola* (*Pantoea agglomerans*) reduced disease severity of *S. sclerotiorum* in growth chambers at 24 °C (Godoy et al., 1990). Spraying of *B. cereus* strain alf-87A reduced ascospore infection of *S. sclerotiorum* on dry peas (Huang et al., 1993). However, to our knowledge, this is the first study to investigate the possible mechanisms involved in phyllosphere biocontrol of Sclerotinia in canola.

Microscopic observation of ascospore germination on canola petals either in the presence or absence of PA-23 indicated that the germination was inhibited in the presence of PA-23. In contrast, complete colonization of petals by *S. sclerotiorum* was observed in the absence of PA-23. Treatment of canola blossoms with PA-23 at a concentration of 10^4 cfu ml^{-1} may inhibit the germination of ascospores either through the production of antimicrobial substances or direct growth on ascospores. Antibiotic production by fluorescent pseudomonads suppresses the activity of pathogen and disease development due to its direct involvement in pathogen inhibition (Handelsman and Stabb, 1996).

The potential for antagonistic bacteria to control plant diseases has been demonstrated in several crops. However, the efficiency of biocontrol agents can be improved through elucidation of the mechanism of action. Plants may be protected against pathogens by way of endogenous defense mechanisms that are triggered in response to the attack of either an insect or pathogen (Heil, 2001). Induced resistance by bacterial antagonists in several crops are associated with the enhancement of lignification and stimulation of host-defense enzymes and synthesis of pathogenesis-related (PR) proteins (Hammerschmidt and Kuc, 1995). Our results suggest that two applications of PA-23 induced resistance against *S. sclerotiorum* infection. PR proteins, chitinase and β-1,3-glucanase inhibits fungal pathogens (Mauch et al., 1988). The enhanced accumulation of PR proteins and oxidative enzymes including chitinase and β-1,3-glucanase by PA-23 in canola leaf tissues may also be responsible for the reduction of *Sclerotinia* infection in pathogen inoculated plants. Since, fungi have chitin and glucan as cell wall components (Sing et al., 1999), increased activity of chitinase and β-1,3-glucanase in canola plants exposed to biocontrol bacteria may prevent establishment of pathogens. Plant growth promoting rhizobacteria (PGPR) strains with induced systemic resistance (ISR) activity can be active against a wide range of pathogens (Raupach and Kloepper, 1998). Prior application of fluorescent pseudomonads strengthens the host cell wall structures and results in the restriction of pathogen invasion of host-plant tissue (Chen et al., 2000). However, a combination of antibiotic production with ISR may act synergistically in restricting the growth and colonization of pathogens. PA-23 induced systemic defense-related enzymes and its produced antibiotics are effective against *Sclerotinia*. Earlier work demonstrated that untreated healthy plants do not have any activity of host-plant resistance genes (M'Piga et al., 1997). Our studies also revealed that application of PA-23 followed by inoculation with *Sclerotinia* ascospores resulted in increased activity of a 34 kDa chitinase.

A major advantage of PGPR is that once systemic resistance is induced, the natural defense mechanisms of the plants are operative for prolonged periods, even when populations of inducing bacteria decline over time (van Loon et al., 1998). Spray application of PA-23, BS6 or DF41 helped to control stem rot of canola by reducing petal infestation of *Sclerotinia* through both direct antimicrobial action and/or induction of plant defense enzymes. In the field efficacy of strains PA-23, BS6, DF41, and E16 varied from year-to-year depending on the existing environmental conditions. However, in general stem rot incidence was significantly reduced by applying PA-23 and BS6 bacteria showing biocontrol promise of Sclerotinia stem rot disease of canola.

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References

Adams, P.B., Ayers, W.A., 1979. Ecology of *Sclerotinia* species. Phytopathology 69, 896–899.

Bardin, S.D., Huang, H.C., 2001. Research on biology and control of *Sclerotinia* diseases in Canada. Can. J. Plant Pathol. 23, 88–98.

Boland, G.J., 1997. Stability analysis for evaluating the influence of environment on chemical and biological control of white mold (*Sclerotinia sclerotiorum*) of bean. Biol. Control. 9, 7–14.

Boller, T., Mauch, F., 1988. Colorimetric assay for chitinase. Methods Enzymol. 161, 430–435.

Chen, C., Belanger, R.R., Benhameu, N., Paulitz, T.C., 2000. Defense enzymes induced in cucumber roots by treatment with plant growth promoting rhizobacteria. Physiol. Mol. Plant Pathol. 56, 13–23.

Davies, J., 1986. Diseases of oilseed rape. In: Scarisbrick, D.H., Daniels, R.W. (Eds.), Oilseed Rape. Mackays of Chantham, Great Britain, pp. 195–236.

De Meyer, G., Capieau, K., Audenaert, K., Buchala, A., Metraux, J.P., Höfte, M., 1999. Nanogram amounts of salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 activate the systemic acquired resistance pathway in bean. Mol. Plant-Microbe Interactions 12, 450–458.

Fiume, M.Z., Cosmetic Ingedient Review Expert Panel, 2003. Final report on the safety assessment of Triacetin. Int. J. Toxicol. 22 (Suppl. 2), 1–10.

Fuller, P., Coyne, D., Steadman, J., 1984. Inheritance of resistance to white mold disease in a diallel cross of dry beans. Crop Sci 24, 929–933.

Gallagher, S., Winston, S.E., Fuller, S.H., Hurre, J.G.R., 1995. Immunoblotting and immunodetection. In: Ausubel, F., Bernt, R., Kingston, E.E., Moore, D.D., Seidman, J.D., Smith, J.A., Struhl, K. (Eds.), Short Protocols in Molecular Biology. John Wiley and Sons Ltd, Chichester, UK, pp. 1040–1048.

Gilmour, G., 2001. Canola growers losing Benlate fungicide. Manitoba Cooperator 58, 1.

Godoy, G., Steadman, J.R., Yuen, G., 1990. Bean blossom bacteria have potential for biological control of white mold disease caused by *Sclerotinia sclerotiorum*. Annu. Rep. Bean. Improv. Coop. 33, 45–46.

Hammerschmidt, R., Kuc, J., 1995. Induced Resistance to Disease in Plants. Kluwer Academic Publishers, Dordsecht, The Netherlands, p. 182.

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

Heil, M., 2001. The ecological concept of induced systemic resistance (ISR). Eur. J. Plant Pathol. 107, 137–146.

Huang, H.C., Kokko, E.G.I., Yanke, J., Phillippe, R.C., 1993. Bacterial suppression of basal pod rot and end rot of dry peas caused by *Sclerotinia sclerotiorum*. Can. J. Microbiol. 39, 227–233.

Inglis, G.D., Boland, G.J., 1990. The microflora of bean and rapeseed petals and the influence of the microflora of bean petals on white mold of bean. Can. J. Plant Pathol. 12, 129–134.

Khan, A., Sutton, J.C., Grodzinski, B., 2003. Effects of *Pseudomonas chlororaphis* on *Pythium aphanidermatum* and root rot in peppers grown in small scale hydrophonic troughs. Biocontr. Sci. Technol. 13, 615–630.

M'Piga, P., Belanger, R.R., Paulitz, T.C., Benhamou, N., 1997. Increased resistance to *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato plants treated with the endophytic bacterium *Pseudomonas fluorescens* strain 63-28. Physiol. Mol. Plant. Pathol. 50, 301–320.

Manitoba Agriculture, F.A.R.I., 2002. Pest management—plant disease. Slcerotinia. Available from http://www.gov.mb.ca/agriculture/crops/diseases/fac07s00.html: [updated Jaunary 2002; cited 25 June 2004]

Martens, J., Seaman, W., Atkinson, G. (Eds.), 1994. Diseases of Field Crops in Canada. The Canadian Phytopathological Society, ON, Canada.

Mauch, F., Mauch-Mani, B., Boller, T., 1988. Antifungal hydrolasses in pea tissue: II. Inhibition of fungal growth by combinations of chitinases and two β-1,3 glucanases. Plant Physiol. 87, 936–942.

McGrath, M.T., 2001. Fungicide resistance in curcurbit powdery mildew: Experiences and challenges. Plant Dis. 85, 236–245.

Nelson, B., 1998. Biology of Sclerotinia. In: Proceedings of the 10th International *Sclerotinia* workshop, 21 Jan. 1998, Fargo, ND, USA. North Dakota State University Department of Plant Pathology, Fargo, ND, pp. 1–5.

Pan, Q., Te, Y.S., Kuc, J., 1991. A technique for detection of chitinases, β-1,3 glucanase and protein patterns after single separation using PAGE or isoelectric focusing. Phytopathology 81, 970–974.

Ramarathnam, R., Fernando, W.G.D., 2003. Characterization of antifungal compounds involved in the biocontrol of Leptosphaeria maculans in canola. Phytopathology 93, S72.

Raupach, G.S., Kloepper, J.W., 1998. Mixtures of plant growth promoting rhizobacteria enhance biocontrol of multiple cucumber pathogens. Phytopathology 88, 1158–1164.

Rose, R.I., 1995. Ecological Effects. USDA IR-4/EPA Minor Use Biopesticide Workshop. Washington, DC, 7–8 November 1995, pp. 50–54.

Sall, J., Lehman, A., 1996. JMP Start Statistics: a guide to statistical and data analysis using JMP® and JMP IN® software. SAS Institute, USA.

Savchuk, S.C., 2002. Evaluation of biological control control of *Sclerotinia scleroiorum* on Canola (*Brassica napus*) in the lab, in the greenhouse, and in the field. Msc. thesis, University of Manitoba, pp. 49–83.

Savchuk, S., Fernando, W.G.D., 2004. Effect of timing of application and population dynamics on the degree of biological control of *Sclerotinia sclerotiorum* by bacterial antagonists. FEMS Microbiol. Ecol. 49, 376–388.

Sing, P.P., Shin, Y.C., Park, C.S., Chung, Y.R., 1999. Biological control of Fusarium wilts of cucumber by chitinolytic bacteria. Phytopathology 89, 92–99.

Slininger, P.J., Burkhead, K.D., Schisler, D.F., Bothast, R.S., 2000. Isolation, identification, and accumulation of 2-acetamidophenol in liquid cultures of the wheat take all biocontrol agent Pseudomonas fluorescens 2-79. Appl. Microbiol. Biotechnol. 54, 376–381.

Tu, J.C., 1997. Biological control of white mould in white bean using *Trichoderma viride*, *Gliocladium roseum* and *Bacillus subtilis* as protective foliar spray. In: Proceedings of 49th International Symposium on Crop Protection. 6 May 1997, Gent. Part IV. Meded. Fac. Landbouwkd. Toegep. Biol. Wet. Univ. Gent, 62, 979–986.

Turkington, T.K., Morrall, R.A.A., 1993. Use of petal infestation to forecast sclerotinia stem rot of canola: the influence of inoculum variation over the flowering period and canopy density. Phytopathology 83, 682–689.

van Loon, L.C., Baker, P.A.H.M., Pieterse, M.J., 1998. Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol. 36, 453–483.

Venette, J., 1998. Sclerotinia spore formation, transport and infection. In: Proceedings of the Sclerotina Workshop. 21 January 1998. Fargo, North Dakota, USA

Viswanathan, R., Samiyappan, R., 1999. Induction of systemic resistance by plant growth promoting rhizobacteria against red rot disease in sugarcane. Sugarcane Technol. 1, 67–76.

Viswanathan, R., Samiyappan, R., 2001. Antifungal activity of chitinases produced by some fluorescent pseudomonads against Colletotrichum falcatum Went. causing red rot disease in sugarcane. Microbiol. Res. 155, 309–314.

Vrije, T.d., Antoine, N., Buitelaar, R.M., Bruckner, S., Dissevelt, M., Durand, A., Gerlagh, M., Jones, E.E., Luth, P., Oostra, J., 2001. The fungal biocontrol agent *Coniothyrium minitans*: production by solid-state fermentation, application and marketing. Appl. Microbiol. Biotechnol. 56, 58–68.

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

Yuen, G.Y., Kerr, E.D., Steadman, J.R., Craig, M.L., 1992. Bacterial biocontrol of white mold disease (*Sclerotinia sclerotiorum*). Annu. Rep. Bean Improv. Coop. 35, 54–55.

Yuen, G.Y., Craig, M.L., Kerr, E.D., Steadman, J.R., 1994. Influences of antagonist population levels, blossom development stage, and canopy temperature on the inhibition of *Sclerotinia sclerotiorum* on dry edible bean by *Erwinia herbicola*. Phytopathology 84, 495–501.

Zdor, R.E., Anderson, A.J., 1992. Influence of root colonizing bacteria on the defense responses in bean. Plant and Soil 140, 99–107.

Zhang, Y., 2004. Biocontrol of Sclerotinia stem rot of canola by bacterial antagonists and study of biocontrol mechanisms involved. Msc. thesis, University of Manitoba, MB, p. 147.

Zhang, Y., Fernando, W.G.D., 2004a. Zwittermicin A detection in *Bacillus* spp. controlling *Sclerotinia sclerotiorum* on canola. Phytopathology 94, S116.

Zhang, Y., Fernando, W.G.D., 2004b. Presence of biosynthetic genes for phenazine-1-carboxylic acid and 2, 4- diacetylphloroglucinol and pyrrolnitrin in *Pseudomonas chlororaphis* strain PA-23. Can. J. Plant Pathol. 26, 430–431.

Zhang, Y., Fernando, W.G.D., de Kievit, T., Berry, C., Daayf, F., Paulitz, T.C., 2006. Detection of antibiotic-related genes from bacterial biocontrol agents using polymerase chain reaction. Can. J. Microbiol. 52, 476–481.