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Preliminary phenotypic and molecular screening for potential bacterial biocontrol agents of *Leptosphaeria maculans*, the blackleg pathogen of canola

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Abstract

Leptosphaeria maculans causes blackleg disease of canola (*Brassica napus* L.). Bacteria isolated from soil, canola stubble and plant parts were assayed for suppression of blackleg. In plate assays, the bacteria isolated from canola stubble had the highest agar-diffusible antifungal activity (75%), which was fungitoxic. In plant cotyledon assays, endophytes had the highest disease suppression. Bacteria with the highest disease suppression in cotyledon assays also had significant disease suppression at the three- to four-leaf stage. PCR screening for bacterial biosynthetic genes, commonly thought to be involved in plant disease suppression, revealed 22 bacteria to be positive for pyrrolnitrin. *Pseudomonas chlororaphis* and *P. aurantiaca* isolates contained the phenazine biosynthetic gene. Three *Bacillus cereus* isolates had the *zmaR* resistance gene. This study generated a novel set of primers specific to the zwittermicin A biosynthetic cluster. The PCR screening has confirmed the presence of genes encoding pyrrolnitrin (55%), phenazine (10%), zwittermicin A biosynthesis (7.5%) and zwittermicin A resistance (7.5%) from the canola phyllosphere and rhizosphere, which seems more widely distributed than genes for 2,4-diacetylphloroglucinol and pyoluteorin.

Keywords: *Antagonistic bacteria, canola, phenazine, pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol, zwittermicin A*

Introduction

Canola, a member of the *Brassicaceae*, is the largest oilseed crop grown in Canada. It has been a significant contributor to the Canadian net trade balance, with annual cash receipts totaling 1.6 billion dollars in 2002, and the acreage almost doubling between 1988 and 1994 in Canada (Statistics Canada 2003). However, with the increased production of oilseed rape, blackleg, caused by *Leptosphaeria maculans* (Desm.) Ces and de Not [anamoph *Phoma lingam* (Tode: Fr./Desm.)] has become a disease of major economic importance and occurs in epidemic proportions in most of the rapeseed producing regions in Australia, Europe and Canada (West et al. 2001).

Several strategies such as crop rotation, stubble management, chemical control, sanitation and resistant cultivars have been advised for blackleg control (Guo et al.

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2005). With the introduction of cultivars with major gene resistance to *L. maculans*, a high potential exists for the increase of the aggressive isolates of *L. maculans*, or evolution of new virulent pathotypes of the pathogen (Mayerhofer et al. 1997). The report on the appearance of the more aggressive Pathogenicity group 3 (PG3) and 4 (PG4) isolates in W. Canada and North Central United States, where PG2 is the predominant group, is a good example of this (Fernando & Chen 2003; Bradley et al. 2005; Chen & Fernando 2005). With respect to chemical control with fungicides, the perceived health and environmental risks of using these chemicals has made the trend grow towards alternate disease management strategies (Jacobsen & Backman 1993). Also, the continuous use of fungicides initiates the evolution of resistant races of the pathogen (Fry et al. 1993). Biocontrol seems to be a viable alternative to be included in an approach, which involves harnessing disease-suppressive microorganisms to improve plant health (Handelsman & Stabb 1996). Bacteria, especially the pseudomonads and bacilli, and their antibiotics have been shown to play a key role in the suppression of plant pathogens in other cropping systems. Some prominent examples are: control of *Gaeumannomyces graminis* var *tritici* in wheat by phenazine produced by *Pseudomonas chlororaphis* PCL1391 (Chin-A-Woeng et al. 1998); control of *Rhizoctonia solani* in cotton by pyrrolnitrin produced by *P. fluorescens* BL915 (Ligon et al. 2000); control of *Thielaviopsis basicola* and *Pythium ultimum* in cotton by pyoluteorin produced by *P. fluorescens* CHA0 (Keel et al. 1992); control of *Gaeumannomyces graminis* var *tritici* in wheat by 2,4-diacetylphloroglucinol (DAPG) produced by *P. fluorescens* Q8r1-96 (Raajmakers & Weller 2001); control of *Phytophthora medicaginis* in alfalfa by zwittermicin A produced by *B. cereus* UW85 (Silo-Suh et al. 1994). The recovery of phenazine-1-carboxylic acid (PCA) from wheat roots and associated rhizosphere soil colonized by *P. fluorescens* 2-79, and the inability of the antibiotic non-producing mutants to suppress disease (Thomashow et al. 1990) are direct evidence for the role of antibiotics in disease suppression. The narrow susceptibility period of canola to blackleg favors the use of biocontrol as a viable disease control strategy. The disease is destructive to the canola crop only when the infection occurs early in plant development from cotyledon to six-leaf growth stage (West et al. 2001). If the plant can be protected during this most susceptible period, disease could be managed to avert yield loss.

The objective of this study was to identify and characterize bacterial agents that will be effective in the phyllosphere biocontrol of *L. maculans*. This study involved the screening for antagonistic, antifungal bacterial biocontrol agents from canola through: (1) plate; (2) plant (cotyledon and three- to four-leaf stage); and (3) PCR-based assays. Preliminary reports have been published (Ramarathnam & Fernando 2003, 2004).

Materials and methods

Bacterial strains and culture conditions

Bacteria were isolated from soil, diseased and non-diseased plant parts (flower, leaf, stem and root tip) and canola stubble collected from field (Savchuk 2002). Each of these samples were suspended in sterile distilled water and sonicated for 30 s. One hundred microlitres of the water suspension was plated on ½-strength nutrient agar (NA; Difco Laboratories, Detroit, MI, USA) plates and incubated at 28°C. Single colonies were selected to ensure pure cultures (Savchuk 2002). To isolate bacterial endophytes, leaves of *B. napus* cvs. Westar and Cresor from different growth stages,

such as cotyledon, rosette, bud and flowering were cut into 1 × 1-cm fragments. The leaf fragments were surface-sterilized using a modified method of Dobranic et al. (1995). The surface sterilized pieces were plated on NA plates, amended with Nystatin (6.7 mg L^{-1}), and incubated at 32°C. Pure cultures of each bacteria were maintained in Luria Bertani broth (LBB), amended with 20% glycerol (Fisher Scientific, Fair Lawn, NJ, USA) and stored at −80°C. Bacteria were streaked from the stock onto Luria Bertani agar (LBA) or NA plates and incubated at 28°C for 24 h. For the plate and plant assays, 5 mL of LBB was inoculated with a bacterial loop and incubated at 28°C for 16–18 h at 180 rpm in an incubator shaker (Jeio Tech SI-600, Seoul-City, South Korea).

Fungal strain and culture conditions

The fungal culture, *L. maculans* (PL 86-12), belonging to Pathogenicity group 2, was stored as concentrated pycnidiospore suspension in sterile water at −20°C. The fungal cultures were initiated by diluting the concentrated stock in sterile distilled water and spreading a few drops onto a V8 agar (200 mL V8 juice, 0.75 g $CaCO_3$, 800 mL distilled water, 17 g agar) plate. The plates were incubated at room temperature under a light bank, until dark pycnidia started forming on the surface of the agar. Pieces of the agar (1 × 1 cm), containing the pycnidia were placed upside down on fresh V8 agar plates. The plates were incubated under light at room temperature, until the mycelial growth reached the periphery of the plates.

Plate inhibition assays

Potato dextrose agar (PDA, Difco) was used for *in vitro* antagonism tests. Five microlitres of O/N bacterial culture were dispensed carefully at four equidistant points along the periphery of the plates, containing PDA. The plates were incubated at room temperature for 24 h. Mycelial plugs of *L. maculans* (5 mm diameter) were cut out from the edges of an actively growing colony and placed mycelial side down on the agar, at the center of the assay plates. The control plates had only the fungal mycelial plug. The plates were incubated under light 25°C and scored for the radial mycelial growth, when the radial growth of the mycelia in the control plates reached the periphery of the plate. The mycelial growth between each of the two opposite bacterial spots was measured. The percentage of mycelial inhibition (%) was calculated using the equation (Fernando & Pierson 1999):

$$R_1 - R_2/R_1 \times 100$$

where, R_1 is maximum radius of mycelial growth on the control plate and R_2 is radius of mycelial growth directly opposite to the bacterial growth.

All treatments had five replicates. The PDA inhibition experiment was repeated once. Due to the large number of bacteria assayed, the analysis was performed by grouping the bacteria based on their source of origin: endophytes, flower, leaf, root tip, soil, stem and stubble.

Plant assays

Cotyledon assays. Cotyledons of *B. napus* cv Westar were used for the assays. The cotyledons were grown in METRO-MIX® contained in S806 'T' inserts that were placed in rectangular trays. The plants were grown in a controlled growthroom and

incubated at 22/18°C day/night and 16/8 h photoperiod (280 μE m^{-2} s^{-1}) with daily watering. The 7–8-day-old seedlings were wounded with forceps, one wound per cotyledon lobe. A 10-μL drop of 16–18 h-old bacterial culture (10^8 cfu mL^{-1}), grown in LBB, was placed on the wound, 24 h prior to inoculation of pycnidiospores of *L. maculans*. A 10-μL drop of pycnidiospores of *L. maculans* (2×10^7 spores mL^{-1}) was placed on each wound site. A drop of Tween 20 was added to 10 mL of pycnidiospore suspension to enhance the spores to be equally distributed on the surface of the leaves. Control treatments included plants treated with LBB or water 24 h prior to the pycnidiospore inoculation. The seedlings were placed in a controlled growthroom and incubated at conditions mentioned above. The true leaves were removed on a regular basis, until cotyledons were scored for the disease levels. Disease severity was scored using an interaction phenotype (IP) scale of 0–9, 10–12 days post-pycnidiospore inoculation, where, 0 = no darkening around the wounds, as in healthy control; 1 = limited blackening around the wound, lesion diameter = 0.5–1.5 mm, faint chlorotic halo may be present, sporulation absent; 3 = dark necrotic lesions, 1.5–3.0 mm chlorotic halo may be present, sporulation absent; 5 = non-sporulating 3–5-mm lesions, sharply limited by dark necrotic margin, may show gray–green tissue collapse as in IP 7 and 9 or dark necrosis throughout; 7 = gray-green tissue collapse, 3–5 mm diameter, sharply delimited, non-darkened margin; 9 = rapid tissue collapse at about 10 days, accompanied by profuse sporulation in large, more than 5 mm, lesions with diffuse margins. The experiment contained five plants per treatment and was repeated once.

Assays at three- to four-leaf stage. Brassica napus cv Westar plants at the three- to four-leaf stage were used for the assay. Cotyledons were raised as mentioned in the previous section and allowed to reach the two-leaf stage, when they were transplanted into 11.5 cm-pots containing soil mixture (2:1:1, soil, sand and peat moss, respectively). The plants were grown in controlled growth rooms under conditions mentioned in the previous section. Each treatment contained 10 plants and two leaves per plant were picked for the assay. Ten wound sites were made on one leaf and the other leaf was left intact. Both bacteria and pycnidiospores were sprayed on wounded and intact leaves. The inoculum concentration, time of application and assay conditions were same as mentioned in the cotyledon assays, except that Tween 20 was added to the bacterial suspension, prior to spraying. The experiment had 10 replicates per treatment and 100 lesions scored per treatment. The experiment was repeated once.

Bacterial identification

Some of the bacteria demonstrating consistent antifungal activity were selected for further identification, using the Microlog® system (Biolog Inc., Hayward, CA). Single colonies were obtained by the streaking method and the following steps were involved in the process of identification: (i) Gram stain (Biolog) rated as Gram-positive or Gram-negative; (ii) bacteria were streaked onto Biolog universal growth (BUG) agar medium (Biolog); (iii) approximate bacterial number were 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 for 32°C for 16–24 h and then read with an automated plate reader (Biolog), assessed visually and identified to genus or species level.

Data analysis

Analysis of variance (ANOVA) and a mean separation test (Fisher's least significant difference), at $P = 0.05$, were performed for interaction within bacterial origin groups, and interaction between bacterial origin groups were tested at 95% confidence limit for the expected mean square. The Analyst procedure of SAS, Version 8.1 (SAS Institute, Cary, NC, USA) was used for the statistical analysis.

PCR analysis

In this study, we designed a primer pair for the detection of the zwittermicin A biosynthetic cluster using the web-software Primer3 (Rozen & Staletsky 2000). A 1218-bp region [GenBank accession # AF155831 (Region: 2630–3847)], an orf2 – proposed to be involved in the biosynthesis of hydroxymalonyl-ACP, which is necessary for the biosynthesis of zwittermicin A (Emmert et al. 2004) – was used to design the primers zwitF2 (5′- TTGGGAGAATATACAGCTCT-3′) and zwitR1 (5′- GACCTTTTGAAATGGGCGTA-3′). The specificity of the primers were checked with a nucleotide to nucleotide BLAST search, and also by including non-related bacterial species in the PCR analysis.

A list of primers used for the detection of antibiotic biosynthetic genes of phenazine, pyrrolnitrin, pyoluteorin, 2,4-DAPG and zwittermicinA is presented in Table I.

Total genomic DNA was isolated from bacterial strains by a cetyltrimethylammoniumbromide (CTAB)-based miniprep protocol (Ausubel et al. 1995). PCR amplifications were performed in a 25-μL reaction mixture containing 20 ng of template DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dATP, dCTP, dGTP and dTTP (Invitrogen Life Technologies, CA, USA), 20 pmol of each primer (50 pmol for primers 677 and 678) (Invitrogen) and 2.0 U of Platinum®Taq (Invitrogen). The following bacterial strains were used as positive controls for the PCR analysis: *P. fluorescens* Pf-5 for pyrrolnitrin and pyoluteorin (Dr. Joyce Loper lab); *P. fluorescens* 2-79 and *P. fluorescens* Q2-87, for phenazine and 2,4-DAPG, respectively (Dr Linda Thomashow lab); and *B. cereus* UW85 for zwittermicin A resistance gene (Dr Jo Handelsman lab). Amplifications were carried out with a PTC-100™ programmable thermal controller. The PCR program for the zwittermicin A biosynthetic primers was: one cycle at 94°C; 45 cycles of 94°C, 52°C for 1 min, 72°C for 2 min; and a final extension of 72°C for 5 min. The PCR programs for the amplification of the genes encoding the respective compounds were followed as mentioned in the relevant literature: pyrrolnitrin (de Souza & Raaijmakers 2003), pyoluteorin (Mavrodi et al. 2001), phenazine (Delaney et al. 2001), 2,4-DAPG (McSpadden Gardner et al. 2001), zwittermicin A resistance gene (Stohl et al. 1999). Eight microlitres of each sample were loaded onto a 1 or 1.5% agarose gel, containing ethidium bromide and electrophoresed in 1 × Tris–borate EDTA (TBE) buffer at 100 V for 1-2 h. The gels were visualized with a UV illuminator and digitally recorded. The bacterial DNA that tested positive with a specific amplification product, were re-amplified with the specific primers and the desired bands were purified with a High Pure PCR product purification kit (Roche Diagnostics Technologies, IN, USA). The amplified products were quantified in agarose gel with the 1 kb-ladder, and were sequenced at the University of Calgary, Biotechnology Lab. The sequences obtained were searched for homology, with sequenced genes in the GenBank database, through the NCBI-Blast search for nucleotides.

Table I. List of primers used in the PCR detection of bacterial antibiotic biosynthetic genes in selected bacterial isolates with biocotrol potential against *Leptosphaeria maculans* on canola.

Antibiotic	Primer	Primer sequence (5′–3′)	Product length (bp)	Target gene	Positive strain	Reference
Phenazine	PHZ1 PHZ2	GGCGACATGGTCAACGG CGGCTGGCGGCGTATTC	1408	*phzF* and *phzA* *phzC* and *phzD*	*P. aureofaciens* 30–84 *P. fluorescens* 2–79	Delaney et al. 2001
Pyrrolnitrin	PRND1 PRND2	GGGGCGGGCCGTGGTGATGGA YCCCGCSGCCTGYCTGGTCTG	786	*prnD*	*P. fluorescens* strain BL915	De Souza and Raaijmakers 2003
Pyoluteorin	PLTBf PLTBr	CGGAGCATGGACCCCCAGC GTGCCCGATATTGGTCTTGACCGAG	773	*pltB*	*P. fluorescens* Pf5	Mavrodi et al. 2001
2,4-Diacetyl phloroglucinol	BPF2 BPR4	ACATCGTGCACCGGTTTCATGATG CCGCCGGTATGGAAGATGAAAAAGTC	535	*phlD*	*P. fluorescens* Q2–87	McSpadden Gardner et al. 2001
ZwittermicinA Resitance gene	677 678	TAAAGCTCGTCCCTCTTCAG ATGTGCACTTGTATGGGCAG	1000	*zmaR*	*B. cereus* UW85	Raffael et al. 1996
ZwittermicinA biosynthetic gene	zwitF2 zwitR1	TTGGGAGAATATACAGCTCT GACCTTTTGAAATGGGCGTA	800	*orf2* – zwittermicin A biosynthetic cluster	*B. cereus* UW85	This study

Results

Plate inhibition assay

One hundred and seventy-two bacteria, including 162 isolated by Savchuk (2002) and 10 that were isolated in this study as endophytes (from different growth stages of cultivars Westar and Cresor (cotyledon through flowering), were assayed for mycelial inhibition of *L. maculans* on PDA. The mycelial inhibition data for the two PDA assays were similar and therefore, pooled together for the analysis. Among the isolates grouped based on their origin, the isolates from stubble had the highest mean blackleg-mycelial inhibition (75%) followed by isolates from stem (59%) and isolates of endophytic (59%) origin. In all the plate inhibition assays, where agar diffusible antifungal activity was observed, the mycelial plugs from the inhibitory plates failed to grow when re-plated on fresh V8 plates. The overall mycelial inhibition mean of the PDA group assays and the number of isolates with >50% mycelial inhibition are presented in Table II.

Plant assays

Cotyledon assay. One hundred and seventy-two isolates were assayed for disease suppression of *L. maculans* on canola cotyledon leaves. While rating cotyledon resistance in canola-blackleg resistance breeding, an IP rating of 3 and lower is considered a resistant reaction. Therefore, bacterial treatments with a similar disease rating were considered as good disease suppressive treatments. Bacteria were grouped based on their origin, as grouped for the PDA assays, and were analyzed for their IP rating. Most of the bacterial treatments were significantly different in their IP rating as compared to the control, which had a disease rating of 9. Bacteria that were isolated as endophytes, from the internal tissues of leaves, had the highest disease suppression (lowest IP rating mean, 2.76), followed by the isolates from stubble (IP = 4.92) and leaf surface (IP = 5.44). The overall IP mean of the individual groups, the number of bacterial treatments with an IP rating less than 3, and the bacteria which were

Table II. Mycelial inhibition of *Leptospaheria maculans* in potato dxtrose agar (PDA) by bacterial isolates obtained from different parts of the canola plant and from soil.

Origin-based grouping	No. of isolates assayed	Mycelial inhibition mean (%)[1]	No. of isolates >50% mycelial inhibition
Stubble	36	75.36[a]	29
Stem	30	59.34[b]	22
Endophytes	10	58.78[b]	8
Root tip	28	54.63[c]	17
Leaf	38	42.9[d]	14
Flower	24	41.77[d]	10
Soil	6	34.87[e]	1
Control	–	0[f]	–

[1] The percent mycelial inhibition data were analyzed using analysis of variance (ANOVA) and a mean separation test (Fisher's least significant difference) was performed at $P = 0.05$. The PDA assays were repeated once. The bacteria were grouped based on their origin for the analysis of the mycelial inhibition on PDA and the data of the respective groups were pooled from two separate experiments. Mycelial inhibition means with the same alphabetical superscript are not significantly different from each other (95% confidence limit).

significantly different in their disease control among their respective groups are presented in Table III.

Assay at three- to four-leaf stage. Bacteria from the cotyledon assays that had an IP rating from 0.2 to 3.4 were tested for blackleg suppression at the three- to four-leaf stage (Table IV). The wound inoculation seems very critical, as no disease lesions were observed when pycnidiospores were sprayed onto intact leaves. Consequently, disease ratings were done on the wounded leaves. Of the 42 bacteria tested, all the bacterial treatments had disease suppressive IP ratings, which were significantly different from that of the control. The bacterial treatments had high disease suppression ratings, ranging from 0 from 1.5.

PCR analysis

Forty bacterial isolates (listed in Table IV) were selected for the PCR analysis based on the following criteria: (1) isolates that exhibited high mycelial inhibition in plates and high disease suppression on cotyledons (DF190 to DF148); (2) isolates that exhibited high mycelial inhibition in plates and low disease suppression on cotyledons (DF25 to DF228); (3) isolates that exhibited low mycelial inhibition in plates and high disease suppression on cotyledons (DF177 to DF153); and (4) isolates that exhibited low mycelial inhibition and low disease suppression on cotyledons (DF115). The results of PCR screening for common antibiotic biosynthetic genes are presented in Table IV. Data of the BLAST search of the purified PCR products of selected isolates are presented in Table V.

The zwit F2/R1 primers yielded an 800 bp amplicon, which on the BLAST search showed high similarity with the zwittermicin A biosynthetic cluster in the GenBank (AF155831; Table V). During the *n*BLAST search, the primer sequence showed

Table III. Mean interaction phenotype (IP) rating of bacterial treatments assayed for the suppression of blackleg on cv. Westar cotyledons.

Origin based grouping	No. of isolates assayed	Mean IP[1]	No. of isolates with IP <3	Bacterial isolates[2]
Endophytes	10	2.76[a]	8	DFE4 – *B. cereus* DFE13 – *B. cereus* DFE11 – *B. pumilus* E16
Stubble	36	4.92[b]	9	DF190 – *P. chlororaphis* Biotype D DF192- *Cellulomonas cellasea*
Leaf	38	5.44[c]	9	DF94, DF114, DF117
Soil	6	5.63[c]	1	DF153
Flower	24	5.69[c]	7	DF121, DF181
Stem	30	6.91[d]	2	DF1
Root tip	28	6.96 [d]	1	DF14 – *P. fluorescens* Biotype G
Control	–	9[e]	–	–

[1] The IP rating data were analyzed using analysis of variance (ANOVA) and a mean separation test (Fisher's least significant difference) was performed at $P=0.05$. The bacteria were grouped based on their origin and the data of the respective groups were pooled from the two experiments. IP Means with the same alphabetical superscript are not significantly different from each other (95% confidence limit).

[2] Bacterial treatments, which had an IP rating <3, and were significantly different, in their disease suppression, from the other isolates in the same group.

Table IV. List of bacteria selected for blackleg disease suppression assays at the three- to four-leaf stage and PCR screening for common bacterial antibiotic biosynthetic genes.

Origin	Isolate no.	Bacteria ID	% Mycelial Inhibition	IP rating		PRN	PYO	PHZ	2, 4-DAPG	ZWIT	zmaR
				Cotyledon Stage	3-4 leaf stage						
Stubble surface	DF190	*P. chlororaphis* BiotypeD	100.0	1.0	0.0	+⋆	+⋆	+⋆			
Stubble surface	DF192	*Cellulomonas cellasea*	100.0	1.0	0.2						
Stubble – 5 cm	DF202	*P. chlororaphis* BiotypeD	100.0	1.8	0.0	+⋆	+⋆	+⋆			
Stubble – 10 cm	DF210	*P. chlororaphis* BiotypeD	100.0	1.8	0.0	+⋆	+⋆	+⋆			
Stubble – 10 cm	DF211	*Rhodococcus fascians*	100.0	3.4	1.1	+					
Stubble – 10 cm	DF225		93.1	2.6	0.3	+					
Leaf	DF118		88.5	3.4	0.0						
Stem	DF1		81.4	2.0	0.0	+					
Cresor bud	DFE16		80.3	1.8	0.0						
Root tip	DF14	*P. fluorescens* BiotypeG	79.0	3.0	0.3						
Flower	DF121		77.8	0.4	0.2						
Cresor cotyledon	DFE12		74.6	2.2	0.6						
Stubble surface	DF217		71.5	1.4	0.3	+					
Westar cotyledon	DFE4	*B. cereus*	69.5	0.6	0.0					+⋆	+⋆
Stubble surface	DF218		64.5	1.8	0.2	+					
Leaf	DF97		63.3	3.2	1.5	+					
Flower	DF181		62.5	1.4	0.0	+					
Cresor cotyledon	DFE13	*B. cereus*	58.3	0.2	0.0					+⋆	+⋆
Cresor cotyledon	DFE11	*B. pumilus*	55.6	2.0	0.8						+⋆
Leaf	DF88		55.5	3.0	0.9						
Westar cotyledon	DFE6		53.4	2.4	0.6						
Flower	DF151	*P. fluorescens*	52.4	2.6	0.5	+			+⋆		
Stubble surface	DF215		51.5	3.4	–	+					
Leaf	DF148	*Stenotrophomonas maltophila*	50.0	3.2	0.6	+					
Soil	DF25$^{\alpha}$		79.1	3.8							
Soil	DF20$^{\alpha}$		76.5	4.0							
Soil	DF17$^{\alpha}$		77.5	4.4							
Stubble surface	DF200$^{\alpha}$	*P. aurantiaca*	100.0	4.6		+⋆	+⋆	+⋆			
Stem	DF2$^{\alpha}$		74.6	4.6		+					
Leaf	DF36$^{\alpha}$		52.3	4.6		+					

Table IV (*Continued*)

Origin	Isolate no.	Bacteria ID	% Mycelial Inhibition	IP rating		PRN	PYO	PHZ	2, 4-DAPG	ZWIT	zmaR
				Cotyledon Stage	3-4 leaf stage						
Westar bud	DFE8$^{\alpha}$	*B. cereus*	68.8	4.8						+*	+*
Root tip	DF13$^{\alpha}$		100.0	5.0							
Stubble – 10 cm	DF212$^{\alpha}$		100.0	5.0		+	+				
Stubble – 10 cm	DF227$^{\alpha}$		100.0	5.0		+					
Stem	DF5$^{\alpha}$		67.5	5.0							
Stubble – 5 cm	DF228$^{\alpha}$		57.8	5.0		+					
Flower	DF177		41.8	3.2	1.3	+					
Westar bud	DFE15		29.8	2.6	1.5						+*
Soil	DF153		21.5	2.2	0.2	+					
Leaf	DF115$^{\alpha}$		10.8	6.0		+					
Flower	DF109$^{\beta}$		54.5	1.6	0.1						
Flower	DF98$^{\beta}$		29.0	2.0	1						
Flower	DF100$^{\beta}$		57.0	3.0	0.7						
Flower	DF120$^{\beta}$		41.8	3.4	1.2						
Flower	DF149$^{\beta}$		18.0	3.8	0.8						
Leaf	DF114$^{\beta}$		84.3	0.8	0						
Leaf	DF94$^{\beta}$		44.3	1.4	1.1						
Leaf	DF137$^{\beta}$		29.3	2.2	0.3						
Leaf	DF95$^{\beta}$		41.3	2.6	1.1						
Leaf	DF87$^{\beta}$		52.8	2.8	1.5						
Leaf	DF129$^{\beta}$		50.5	2.8	0.8						
Leaf	DF96$^{\beta}$		53.8	3.2	1.1						
Leaf	DF67$^{\beta}$		62.8	3.4	0.7						
	DF187$^{\beta}$		69.3	1.4	0.4						
Stubble – 5 cm	DF205$^{\beta}$		100.0	1.4	0.2						
Cresor bud	DFE3$^{\beta}$		–	3.0	0.7						
	Control		0.0	9.0	9.0						

$^{\alpha}$ Bacteria screened in plate, cotyledon and PCR assays, but not in the three- to four-leaf stage assays. $^{\beta}$ Bacteria screened in plate, cotyledon and three- to four-leaf stage assay, but not in the PCR assays. PRN, pyrrolnitrin; PYO, pyoluteorin; PHZ, phenazine; DAPG, 2,4-diacetylphloroglucinol; ZWIT, zwittermicin A; *zmaR,* zwittermicin A resistance gene. * PCR products that were purified, sequenced and searched for homology, with sequenced genes in the GenBank database, through the NCBI-Blast search for nucleotides.

Table V. Blast results of the sequenced products obtained from PCR amplification using gene-specific primers for biosynthetic genes of common bacterial antibiotics.

Antibiotic	Isolate	Primer	Product size (bp)	GenBank Accession no.	Obtained GenBank Match	Score	*E* value
Phenazine	*P. chlororaphis* (DF190, DF202, DF210)	PHZ1/PHZ2	1408	L48339	*phzFABCD* genes of *P. aureofaciens*	1651	0
	P. aurantiaca (DF200)			L48339	*phzFABCD* genes of *P. aureofaciens*	1639	0
Pyrrolnitrin	*P. chlororaphis* DF190	PRND1/PRND2	786	U74493	*prnABCD* genes of *P. fluorescens*	345	$8\ e^{-92}$
Pyrrolnitrin	*P. aurantiaca* DF200	PRND1/PRND2	786	U74493	*prnABCD* genes of *P. fluorescens*	76	$2\ e^{-11}$
Zwittermicin A	*B. cereus* (DFE4, DFE8, DFE13)	ZwitF2/ZwitR1	800	AF155831	*orf2*- biosynthetic cluster of *B. cereus*	1551	0
Zwittermicin A Resistance protein	*B. cereus* (DFE4, DFE8, DFE13)	677/678	1000	U57065	*zmaR* resistance gene *B. cereus*	1739	0
Pyoluteorin	*P. chlororaphis* (DF190, DF202, DF210) *P. aurantiaca* DF200	PLTBf/PLTBr	773	AF081920	*P. aeruginosa papB* gene (AJ277639)*	391	e^{-105}
2,4-DAPG	*P. fluorescens* DF151	BPF2/BPR4	~600	U41818	Insignificant	–	–
Zwittermicin A Resistance protein	Isolate DFE11, DFE15	677/678	600	U57065	No match in GenBank		

*GenBank Accession number AJ277639 for the gene sequence of *papB* of *P. aeruginosa*.

100% identity (E score – 0.2) to *Bacillus thuringenisis* serovar *kurstaki* – putative malonyl CoA-acyl carrier protein transacylase (AF235003.2) and *Bacillus cereus* zwittermicin A biosynthetic gene cluster (AF155831.2). Also, during the PCR analysis, the primers did not amplify the 800 bp amplicon from DNA of *P. chlororaphis*, *P. fluorescens* and *B. amyloliquefaciens,* to name a few, and other Gram-negative bacteria included in the analysis. Of the forty bacterial DNA templates that were screened for the presence of the *prnD* gene, 22 isolates (55% of the total bacteria tested) yielded the specific 786-bp amplification product (Table IV), along with the positive control *P. fluorescens* Pf-5. Four amplified products from *P. chlororaphis* BiotypeD (DF190, DF202 and DF210) and *P. aurantiaca* (DF200) were sequenced, with the products from the *P. chlororaphis* yielding higher similarity with the GenBank# U74993 (*prnABCD* genes of *P. fluorescens)* than the *P. aurantiaca* product (Table V). For phenazine, isolates DF190, DF200, DF202 and DF210 (10% of bacteria screened) yielded the specific 1408-bp amplification product (Table IV), as seen in the positive control *P. fluorescens* 2-79. The products when sequenced and searched with the blast nucleotide search yielded very high similarity with the *phzIRABCDEFGH* genes of *P. chlororaphis* (AF195615; Table V). Screening for the presence of the *pltB* gene of the pyoluteorin biosynthetic cluster, yielded a ~773-bp band in five bacteria (Table IV), along with the positive control *P. fluorescens* Pf-5. The amplification product of the isolates DF190, DF200, DF202 and DF210, when sequenced and blast searched yielded very high similarity with the *P. aeruginosa papB* gene (AJ277639) and not the pyoluteorin biosynthetic cluster (Table V). None of the isolates, except DF151 and the positive control (*P. fluorescens* Q2-87) yielded the 535-bp band, representing a portion of the *phlD* of the 2,4-DAPG biosynthetic cluster. The amplification product of isolate DF151, when sequenced and searched in the GenBank did not yield any significant match (data not shown). Isolates DFE4, DFE8 and DFE13 yielded a 1-kb amplification product along with the positive control *B. cereus* UW85, while screening for the presence of *zmaR* – the zwittermicin A resistance gene. Isolates DF13, DF20, DF25, DF153, DF177, DFE11 and E15 yielded a distinct 600-bp product (Table IV). The products of isolates DFE4, DFE8, DFE13, DFE11 and DFE15 were sequenced and searched in the GenBank using the nucleotide blast. The 1-kb product of isolates DFE4, DFE8 and DFE13, all *B. cereus* isolates, yielded very high similarity with the *zmaR* resistance gene of *B. cereus* (U57065). But, the 600-bp products of DFE11 and DFE15 did not yield any significant match in the GenBank (Table V).

Discussion

This study presents the results of a large-scale screening of bacteria, isolated from canola, for the suppression of the blackleg pathogen, *L. maculans*. Bacteria isolated from the surface of diseased and non-diseased parts of canola, soil, stubble and from internal leaf tissue, showed very high potential for the use as biocontrol agents (BCA) against *L. maculans*. The bacteria inhibited mycelial growth in plates and prevented infection by pycnidiospores of *L. maculans* in plant assays. Also, several of the bacteria tested positive for biosynthetic genes of antifungal antibiotics phenazine, pyrrolnitrin and zwittermicin A. Some bacteria (DF14, DF88, DF118, DF121, DF192, DFE6 and DFE12), though tested negative for the antibiotics screened (Table IV), exhibited significant agar-diffusible antifungal activity in plates and high disease suppression in

plants, suggesting their potential as sources of other antifungal mechanisms or presence of novel antibiotics. This needs to be investigated further. The origin of these bacteria from canola, a member of *Brassicaceae*, further emphasizes the importance of cruciferous plants as a reservoir of antagonistic bacteria, as suggested by Berg et al. (2002) and Johansson and Wright (2003). Berg et al. (2002) reported a high number of disease-suppressive, antagonistic bacteria from oilseed rape (*B. napus* var. *oleracea*). Johansson and Wright (2003), in their search for potential biocontrol agents effective against wheat seedling blight, isolated a high number of disease-suppressive bacteria from cruciferous plants. Similarly in this study, we isolated from canola a high percentage of disease suppressive bacteria, out of which 57% showed >50% inhibition of the fungal mycelial growth and 24% displayed a disease suppressiveness IP rating of 0–3.4.

Competence of the antagonist and synchronization of its activity, in time and space, with the pathogen are key factors that determine the efficiency of a BCA (Folman et al. 2003). In our study, we considered testing the bacteria *in vivo* at the most crucial stages of blackleg infection, i.e., the cotyledon and the three- to four-leaf stage of canola, which on infection enables the movement of the fungus to the base of the plant, leading to cankering and death of the stem. This narrow period of susceptibility of the host enables the use of bacterial BCAs, which favor a short duration for establishing their numbers and survival, to antagonize the pathogen at its course of primary infection of the plant. Phyllosphere bacteria could act as potential resource for biocontrol on leaves, as they have naturally evolved to compete and survive in the harsh conditions that prevail in the phyllosphere. Twenty-nine isolates, of the 43 bacteria that had high disease suppression in plant assays, were obtained from the surface of flowers, stem, leaves, and internal tissues of the leaves. The role of endophytic bacteria in biological control has been previously demonstrated (Chen et al. 1995; Kloepper et al. 1992). In our study *Bacillus* spp, which are known for their antifungal antibiotics (Edwards et al. 1994) and environmental stress-resistant endospores (Sadoff 1972), dominated the endophyte assemblage. These features seem to enable them to colonize the phyllosphere, where they were able to suppress the pycnidospores of the blackleg pathogen. Stubble in the soil is a rich organic source of nutrients that serve as microenvironments, where microorganisms compete with one another for nutrients and space, and in due course produce secondary metabolites, such as antibiotics, which help in the elimination of other microorganisms.

Antifungal antibiotics of microbial origin, which are synthesized biologically, have been demonstrated not only to have specific activity against the target pathogens but also to be generally biodegradable (Yamaguchi 1996), thus overcoming the concern with residual effects of synthetic fungicides. In our study, 21 of the 39 bacteria, isolated from stubble (at different depths in the soil), had 80–100% inhibition of the radial mycelial growth of *L. maculans*, through agar-diffusible antifungal activity. Therefore, *in vitro* assay is as a quick indicator of the mode of antifungal action, especially when it involves antibiosis. The bacteria isolated from the stubble not only showed fungal inhibition in plates, but 11 of them had high blackleg suppression (IP ratings <3.4) in plant assays. This seems to indicate the possible production of the antifungal antibiotics in the phyllosphere as the cause of blackleg suppression.

Selection and identification of antibiotic producing bacteria from natural environments through random isolation and screening procedures is time-consuming and

laborious (de Souza & Raaijmakers 2003). The partial or complete cloning and sequencing of the antibiotic biosynthetic and regulatory genes has facilitated the development of specific primers and probes that can be used for the PCR-based detection of specific antibiotic-producing bacteria (de Souza & Raaijmakers 2003). These include zwittermicin A produced by *B. cereus*, and 2,4-DAPG, pyrrolnitrin, pyoluteorin and phenazine produced by various *Pseudomonas* species. In our screening, 18 isolates, other than the three isolates of *P. chlororaphis* and one isolate of *P. aurantiaca,* tested positive for the presence of pyrrolnitrin biosynthetic genes. This result is in support of earlier findings on the wide distribution of pyrrolnitrin and its wide range of producers (Hammer et al. 1999). Isolate DF1, with a mycelial inhibition of 81%, IP rating of 2 and a positive result for the pyrrolnitrin screening could be a pyrrolnitrin-producer and a potential BCA of blackleg. Similarly, isolates DF190, DF202, DF210 (identified as *P. chlororaphis* BiotypeD) and isolate DF200 (identified as *P. aurantiaca*), that tested positive for both phenazine and pyrrolnitrin can be considered as potential BCAs, owing to their multiple antibiotic mechanisms. These isolates showed significant inhibition of the pathogen both in the plate assays (100%) and cotyledon assays (IP <2). Being native colonizers of the stubble, these isolates may have potential in inhibiting the fungus on this substrate, and prevent the formation of pathogen's reproductive structures. Absence of the pyoluteorin and 2,4-DAPG producers in the screening, further supports earlier finding on the limited distribution of these genes and co-occurrence of their biosynthetic pathways only in a very specific group of 2,4-DAPG producers (Mavrodi et al. 2001). The 800-bp product obtained during screening for pyoluteorin, from the *P. chlororaphis* and *P. aurantiaca* strains, showing high homology to the *papB* gene of *P. aeruginosa*, which encodes a putative autotransporter protein, could be of some significance towards mechanisms involved in the transport of antibiotics to the outside of the cell. This proposition is based on the fact that genes involved in the antibiotic biosynthesis, modification and export pathways are clustered with the genes encoding self-resistance (Milner 1996). Further, the 600-bp products from the non-Bacillus strains could be of significance, even though they did not have a match in the GenBank, as these isolates demonstrated resistance to zwittermicin A (data from a separate study). The role of these products towards resistance to zwittermicin A needs to be investigated.

This study has helped in identifying bacteria that possess the biosynthetic genes for common, well-characterized antibiotics, which could be potential BCAs against the blackleg pathogen. Other bacterial isolates, showing a strong antifungal activity, were identified, and may represent a potential source of new antifungal compounds. These findings also suggest that alternative mechanisms of disease suppression, such as competition for nutrients, lytic enzymes and siderophore production, may contribute to the expression of antifungal properties.

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