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The role of antibiosis and induced systemic resistance, mediated by strains of *Pseudomonas chlororaphis*, *Bacillus cereus* and *B. amyloliquefaciens*, in controlling blackleg disease of canola

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Abstract Antibiotic-producing *Pseudomonas chlororaphis* strains DF190 and PA23, *Bacillus cereus* strain DFE4 and *Bacillus amyloliquefaciens* strain DFE16 were tested for elicitation of induced systemic resistance (ISR) and direct antibiosis in control of blackleg in canola caused by the fungal pathogen *Leptosphaeria maculans*. Inoculation of bacteria 24 h and 48 h prior to the pathogen was crucial for disease control. In systemic induction studies, the bacteria and culture extracts had lower but significant suppression of the blackleg lesion. When inoculated at the same wound site as the pathogen pycnidiospores, the bacterial culture extracts had significantly higher reduction of blackleg lesion development. However, localized plant defense-related enzyme activity at the site of inoculation was not induced by all the bacteria. Direct antifungal activity at the infection site seems to be the dominant mechanism mediating control of *L. maculans*. A Tn*5*-*gacS* mutant of strain PA23 exhibited a complete loss of antifungal and biocontrol activity, which was restored upon addition of the *gacS* gene in trans. Interestingly, a phenazine-minus derivative of PA23 that produces elevated levels of pyrrolnitrin exhibited the same or higher blackleg disease suppression compared to the wild type. These findings suggest that direct antifungal activity, possibly mediated by pyrrolnitrin, and some low level of induced systemic resistance is involved in *P. chlororaphis* biocontrol of blackleg.

Keywords Biocontrol · Induced systemic resistance · Antibiosis · Tn*5* mutant · Pyrrolnitrin

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Introduction

In addition to direct antagonism, competition and hyperparasitism, non-pathogenic bacteria control disease through indirect mechanisms, such as induced systemic resistance (ISR) in the host plant. Bacterial-mediated ISR involves elicitation of the ISR pathway, generation and translocation of the ISR signal, followed by ISR signal transduction leading to ISR-related gene expression and resistance (Pieterse et al. 2001). Even though the full range of inducing agents produced by bacteria is not yet known, among *Pseudomonas* species, the compounds known to elicit ISR are the fluorescent siderophore pseudobactin, the

outer membrane lipopolysaccharide (LPS), and the flagella of *P. putida* WCS358 (De Vleesschauwer et al. 2007; Bakker et al. 2003; Meziane et al. 2005). In addition, the antifungal antibiotics 2,4-diacetylphloroglucinol and phenazine-1-carboxylic acid have been shown to induce ISR. Audenaert et al. (2002) demonstrated that the phenazine compound, pyocyanin, and a siderophore, pyochelin, produced by *P. aeruginosa* strain 7NSK2 both contributed to the ability of this isolate to induce systemic resistance in tomato against *Botrytis cinerea*. In *Arabidopsis thaliana*, ISR by *P. fluorescens* strain CHA0 depends on the production of the antibiotic 2,4-diacetylphloroglucinol (Iavicoli et al. 2003). Massetolide A, a cyclic lipopeptide produced by *P. fluorescens* SS101, was effective in preventing infection of tomato leaves by *P. infestans* and significantly reduced the expansion of existing late blight lesions. Purified massetolide A provided significant control of *P. infestans*, both locally and systemically via induced resistance (Tran et al. 2007). Among the lipopeptide antibiotics produced by *Bacillus* spp., treatment of potato tuber cells with purified fengycins resulted in the accumulation of some plant phenolics involved in or derived from phenylpropanoid metabolism (Ongena et al. 2005). Moreover, in a study by Ongena et al. (2007), purified surfactin of *B. subtilis* S499 was shown to induce systemic resistance against *B. cinerea* in bean and tomato plants. In tomato cells, key enzymes of the lipoxygenase pathway appeared to be activated in resistant plants following induction by surfactin overproducers. With respect to canola, a double application of *P. chlororaphis* strain PA23 on canola petals induced the activity of chitinase and β-1,3 glucanase leading to the suppression of *Sclerotinia sclerotiorum* (Fernando et al. 2007).

Identifying the different mechanisms of biocontrol is important because it may provide a means of attacking pathogens with a broader spectrum of microbial weapons. It should also enable the design of appropriate combinations of strains, for example bacteria with bacteria or bacteria with fungi, for optimal biocontrol (de Boer et al. 1999; Olivain et al. 2004). Blackleg, caused by *Leptosphaeria maculans* (Desm.) Ces & De Not (anamorph, *Phoma lingam* (Tode:Fr./Desm.)), is an economically important disease of canola (*Brassica napus*) in Canada and worldwide. Blackleg is a serious problem in all canola production areas of the world causing severe epidemics, which have threatened the existence of the canola industry. The disease is endemic in countries canola/rapeseed is grown except China. We analyzed four potential antagonistic bacterial strains, *P. chlororaphis* strains PA23 and DF190, both phenazine and pyrrolnitrin producers (Zhang et al. 2006; Ramarathnam 2007), and *B. cereus* strain DFE4 and *B. amyloliquefaciens* strain DFE16, surfactin, iturin A and bacillomycin D producers (Ramarathnam 2007), for their ability to induce systemic resistance towards *L. maculans* in canola. These antibiotic producing bacteria have been demonstrated to inhibit *L. maculans* under in vitro, greenhouse and field conditions (Ramarathnam and Fernando 2006; Ramarathnam 2007). The objectives of this study were: (i) to determine the critical time of inoculation of the antagonistic bacteria for blackleg suppression, (ii) to determine whether bacterial cells and culture extracts (in water:methanol) are able to induce systemic resistance for blackleg suppression, (iii) to determine the ability of bacteria to induce plant defense-related enzyme activity at the point of inoculation of the bacteria and the pathogen, and (iv) to investigate the role of antibiosis in blackleg disease suppression through analysis of antibiotic-deficient mutants.

Materials and methods

General growth conditions

Bacteria were routinely stored at −80°C and streaked onto a Luria–Bertani agar (LBA) followed by incubation at 28°C for strains PA23 and DF190 (*Pseudomonas* strains) and 32°C for strains DFE4 and DFE16 (*Bacillus* strains). For the plant assays, 5 ml of LB broth was inoculated with bacteria and incubated at 28°C (PA23 and DF190) and 32°C (DFE4 and DFE16) for 16–18 h at 180 rev min^{-1} in an incubator shaker. The bacterial cultures were transferred to a 15-ml centrifuge tube and pelleted at 7,000 rpm for 15 min. The bacterial pellets were washed once and re-suspended in sterile distilled water and placed on ice until time of inoculation. Ten microlitres of the bacterial suspension (1×10^8 CFU ml^{-1}) was used as inoculum in the plant assays.

The fungal isolate *L. maculans* (PL 86-12) was stored as a concentrated pycnidiospore suspension in sterile water at −20°C. The inoculum was prepared

by thawing and adding a few drops of the stock to sterile distilled water. The spore concentration was adjusted to 2×10^7 spores ml^{-1}. Ten microlitres of the pycnidiospore suspension was used as the fungal inoculum in the plant 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 growth room 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 days old seedlings were wounded with forceps, one wound per cotyledon lobe. All the assays were carried out at the seedling stage.

Effect of time of inoculation of the bacteria on the suppression of pycnidiospores of *L. maculans*

Bacterial strains PA23, DF190 and DFE4 were chosen for this analysis. Ten microlitres of the bacterial suspension (1×10^8 CFU ml^{-1}) and a 10-μl suspension of pycnidiospores (2×10^7 spores ml^{-1}) were used as inoculum. Both the bacteria and the pathogen were inoculated in the same wound spot (local/single point inoculation (SPI)). The following are the different treatments, which indicate the sequence of inoculation of the bacteria and the pathogen:

1. Bacteria inoculated 24 h prior to inoculation of the pathogen.
2. Bacteria inoculated 48 h prior to inoculation of the pathogen.
3. Bacteria and pathogen inoculated at the same time (co-inoculation).
4. Pathogen inoculated 24 h prior to inoculation of bacteria.
5. Pathogen inoculated 48 h prior to inoculation of bacteria.
6. Control—Sterile water inoculated instead of the bacteria along with the pathogen, following the same sequence of inoculation.

The seedlings were left on the counter to allow the pycnidiopore suspension to dry (6–8 h) before placing them in a controlled growth room for incubation under the conditions mentioned above. Emerging true leaves were removed to maintain the nutrients at the cotyledons. 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; 7 = gray-green tissue collapse, 3–5 mm diameter, sharply delimited, non-darkened margin; 9 = rapid tissue collapse at about ten days, accompanied by profuse sporulation in large, more than 5 mm, lesions with diffuse margins. Each treatment consisted of 12 cotyledon plants and the experiment was repeated once.

Effect of split inoculation of bacterial cells on the suppression of *L. maculans* on cotyledons

Bacterial strains PA23, DF190, DFE4 and DFE16 were tested for their ability to induce ISR for suppression of *L. maculans*. A 10-μl aliquot of the bacterial suspension (1×10^8 CFU ml^{-1}) was inoculated onto one wounded cotyledon. Ten microlitres of the pathogen pycnidiospore suspension (2×10^7 spores ml^{-1}) was placed 24 h post-bacterial inoculation on the other cotyledon that was freshly wounded. The seedlings were left on the counter to allow the inoculation (pycnidiopore suspension) to dry (6–8 h) and then placed in a controlled growth room and incubated under the conditions mentioned earlier. Disease severity was scored as described above. Each treatment consisted of 12 cotyledon plants and the experiment was repeated once.

Effect of split and local inoculation of culture extracts on the suppression of *L. maculans*

Bacterial strains PA23 (phenazine and pyrrolnitrin producer) and DFE4 (iturin A, bacillomycin D and surfactin producer) were chosen for this experiment. The split inoculation was performed to check if culture extracts were capable of inducing ISR. The local inoculation (SPI) was performed to check if the culture extracts had antifungal activity that could suppress the pathogen pycnidiospores. Extracts of 1–5 days old cultures were tested for pathogen

suppression in both types of inoculations, as antifungal metabolites accumulate in the broth over a period of time (2–5 days post inoculation of bacteria). A sample (100 µl) from overnight starter cultures of PA23 and DFE4 was used to inoculate tubes containing 10 ml of LB broth. On days 1, 2, 3, 4 and 5, bacterial cells were removed by centrifugation and the cell-free supernatant was extracted with an equal volume of chloroform. The chloroform extracts were dried under a nitrogen stream and re-suspended in 5 ml water:methanol (50:50) mixture. The control was plain LB broth extracted and suspended in water:methanol mixture. For the SPI, 10 µl of the culture extract was inoculated on a wounded Westar cotyledon leaf and 10 µl of pathogen pycnidiospore suspension (2×10^7 spores ml^{-1}) was inoculated in the same spot 24 h later. For the split inoculation, 10 µl of the culture extract was inoculated on one of the wounded Westar cotyledon leaves, while 10 µl of pathogen pycnidiospore suspension was inoculated on the other freshly wounded cotyledon leaf 24 h later. The seedlings were left on the counter to allow the pycnidiospore suspension to dry (6–8 h) and were handled for disease rating, as described previously. Each treatment consisted of 12 cotyledon plants and the experiment was repeated once.

Assays for induction of plant defense-related enzyme activity

To investigate whether localized suppression of the pathogen was due to the direct antifungal activity or localized induction of plant defense-related enzyme activity by the bacteria, one week old Westar cotyledons were inoculated and assayed for enzyme activity. The treatments included: (1) bacteria alone, (2) bacteria inoculated with the pathogen 24 h post-bacterial inoculation, (3) pathogen alone, and (4) uninoculated control (water). The bacterial and pathogen inoculum was prepared as mentioned earlier. Ten microlitres each of the bacterial (1×10^8 CFU ml^{-1}) and pycnidiospore (2×10^7 spores ml^{-1}) suspension was used as the inoculum. Twelve cotyledons per treatment were collected at time intervals of 0, 24, 48, 72, 96 and 120 h post-bacterial inoculation for treatment 1 and at the same time intervals post-pathogen inoculation for treatments 2, 3, and 4. The samples were collected in plastic bags and stored in −80°C. The cotyledon samples were removed from −80°C and homogenized with liquid nitrogen using a cold mortar and pestle. The powdered samples were stored at −80°C until assayed.

Chitinase assay

One gram of the powdered cotyledon tissue was ground in 1 ml of 0.1 M sodium citrate buffer (pH 5) at 4°C. The homogenate was transferred to a 1.5 ml Eppendorf centrifuge tube and centrifuged at 4°C for 10 min at 10,000*g*. The chitinase activity was determined following the colorimetric method of Boller and Mauch (1988). The chitinase activity which was estimated by the release of *N*-acetyl-glucosamine (GlcNac) from the substrate colloidal chitin was expressed as nmol of equivalent GlcNac min^{-1} mg^{-1} protein.

β-1,3 Glucanase activity

One gram of the powdered cotyledon tissue was ground with 1 ml of 0.05 M sodium acetate buffer (pH 5) at 4°C. The homogenate was allowed to react with 0.4% laminarin (EC2327124, Sigma) for 10 min at 40°C. The colorimetric estimation of the release of a reducing glucose equivalent from laminarin was performed as described by McCleary and Shameer (1987), and β-1,3 glucanase activity was expressed as µg of equivalent glucose min^{-1} mg^{-1} of protein.

Peroxidase activity

One gram of the powdered cotyledon tissue was ground in 1 ml of 0.1 M phosphate buffer (pH 7) using a cold pestle and mortar (4°C). The homogenate was transferred to a 1.5 ml centrifuge tube and centrifuged at 15,000*g* at 4°C for 15 min, and the supernatant was immediately used for the assay. Peroxidase activity was determined using pyrogallol (EC210-762-9 Sigma) as the hydrogen donor and measuring the rate of color development colorimetrically at 420 nm at 30 s intervals for 3 min (Hammerschmidt et al. 1982). The peroxidase activity was expressed as change in absorbance min^{-1} mg^{-1} protein.

Biocontrol of *L. maculans* using Tn*5* mutants of *P. chlororaphis* strain PA23

Several Tn*5* mutant derivatives of *P. chlororaphis* strain PA23 (Poritsanos 2005) were examined for their ability to inhibit *L. maculans* infection in canola.

These strains include wild-type PA23 (produces phenazines, pyrrolnitrin, autoinducer and siderophores, protease and lipase); *gacS* mutant PA23-314 (deficient in the production of phenazine, pyrrolnitrin, autoinducer, and extracellular enzymes); complemented mutant PA23-314 carrying *gacS* in trans on plasmid pUCP23 (PA23-314-G produces wild-type levels of phenazine, pyrrolnitrin, autoinducer, and extracellular enzymes; Poritsanos et al. (2006)); *phzE* mutant PA23-63 harboring a Tn*5* insertion in the phenazine biosynthetic operon (deficient in the production of phenazines; wild type levels of all other exoproducts (Selin et al. 2010)). Strains PA23 (pUCP23) (PA23-P) and PA23-314-(pUCP23) (PA23-314-P) were used as vector controls. Bacteria were cultured in LB broth amended with the following antibiotics: PA23-wild type—rifampicin (25 µg ml^{-1}); strains PA23-314 and PA23-63—tetracycline (15 µg ml^{-1}); strains PA23 (pUCP23), PA23-314 (pUCP23) and PA23-314 (pUCP-*gacS*)—gentamicin (25 µg ml^{-1}). Westar cotyledons, 7–8 days old, were wound inoculated with 10 µl of bacterial suspension (1×10^8 CFU ml^{-1}) followed by 10 µl of pycnidiospore suspension (2×10^7 spores ml^{-1}) 24 h post-bacterial inoculation. The growth and incubation conditions were the same as mentioned earlier. The plants were scored 10–12 days post-pycnidiospore inoculation. Each treatment consisted of 12 cotyledon plants and the experiment was repeated twice.

Data analysis

For the plant assays, analysis of variance (ANOVA) and a mean separation test (Fisher's Least Significant Difference; at $P = 0.05$) were performed, using The Analyst procedure of SAS, Version 8.1 (SAS Institute, Cary, NC, USA).

Results

Effect of time of bacterial inoculation on the suppression of pycnidiospores of *L. maculans*

Bacteria were inoculated at 24 h and 48 h pre- and post-pycnidiospore inoculation as well as together with pycnidiospores (co-inoculation) and then analyzed to determine how fungal inhibition was impacted. The pre-pycnidiospore inoculation of the bacteria at 24 h and 48 h was the most effective for the suppression of *L. maculans* and prevention of blackleg lesions on cotyledons (Fig. 1). The pre-pycnidiospore inoculation of the bacterial strains DF190, PA23 and DFE4 had IP rating values <2, and were significantly different and lower than the co-inoculation, post-pycnidiospore bacterial inoculation and control treatments (Fig. 1). The IP ratings for the co-inoculation, post-pycnidiospore bacterial inoculation and the respective control treatments ranged from 6.2 to 8.9 (Fig. 1).

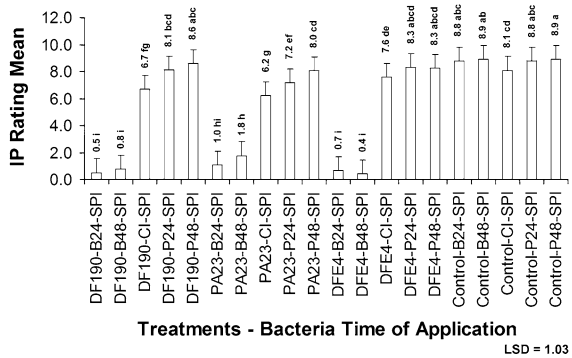

Fig. 1 Effect of time of bacterial inoculation on the suppression of *Leptosphaeria maculans* pycnidiospores on cotyledon leaves of canola cultivar Westar. *Pseudomonas chlororaphis* strains DF190 and PA23, and *Bacillus cereus* strain DFE4, were inoculated 24 and 48 h pre-pycnidiospore inoculation (B24 and B48, respectively), co-inoculated with pycnidiospore (CI), and 24 h and 48 h post-pycnidiospore inoculation (P24 and P48, respectively). Single-point inoculation (SPI) was carried out in all treatments. Disease was scored 10–12 days post pathogen inoculation. Analysis of variance and mean separation testing (Fisher's Least Significant Difference; $P = 0.05$) were performed ($F_{19,460} = 159.70$, $P < 0.0001$). Standard error bars are attached to the means

Effect of split inoculation of bacterial cells on suppression of *L. maculans* on cotyledons

Bacterial strains DF190, PA23, DFE4 and DFE16 were tested for their ability to stimulate ISR, when split inoculated on a cotyledon different from that of the pathogen inoculated cotyledon. The split inoculated bacterial treatments resulted in lower disease reduction. However, the disease reduction was significantly different than the control, with IP ratings

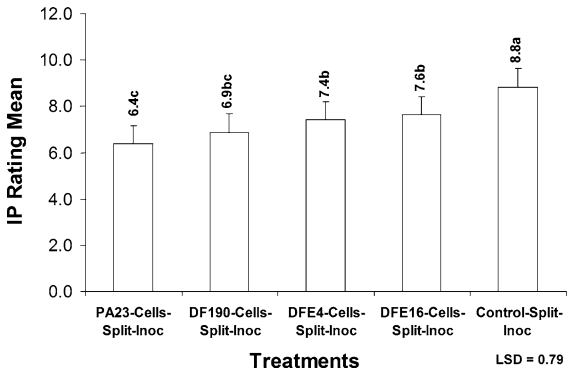

Fig. 2 Effect of split inoculation of bacterial cells on the suppression of *Leptosphaeria maculans* pycnidiospores on cotyledon leaves of Westar. Bacterial cells were inoculated on one cotyledon leaf and 24 h later the pathogen pycnidiospores were inoculated on the other cotyledon leaf (split inoculation). Bacterial isolates *Pseudomonas chlororaphis* strain DF190, *Bacillus cereus* strain DFE4 and *B. amyloliquefaciens* strain DFE16 were analyzed. Disease was scored 10–12 days post pathogen inoculation. Analysis of variance and mean separation testing (Fisher's Least Significant Difference; $P = 0.05$) were performed ($F_{4,115} = 10.7$, $P < 0.0001$). Standard error bars are attached to the means

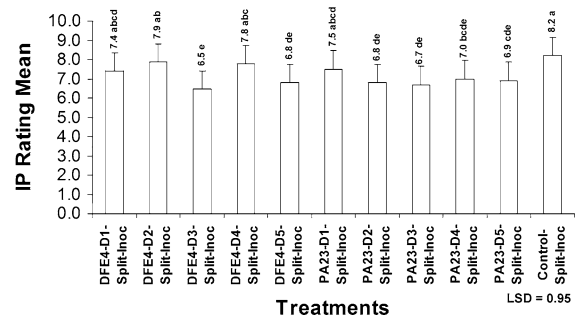

Fig. 3 Effect of split inoculation of culture extracts on the suppression of *Leptosphaeria maculans* pycnidiospores on Westar cotyledons. Culture extracts of *Pseudomonas chlororaphis* strain PA23 and *Bacillus cereus* strain DFE4 were inoculated on one cotyledon and 24 h later pathogen pycnidiospores were inoculated on the other cotyledon (split inoculation). Extracts were generated from 1, 2, 3, 4 and 5-day-old bacterial cultures. Disease was scored 10–12 days after pathogen inoculation. Analysis of variance and mean separation testing (Fisher's Least Significant Difference; $P = 0.05$) were performed ($F_{10,253} = 2.74$; $P < 0.0032$). Standard error bars are attached to the means

ranging from 6.4 to 7.6 (Fig. 2). The pathogen control had an IP rating of 8.8.

Effect of split and local inoculation of culture extracts on the suppression of *L. maculans*

The effect of split (ISR) and local inoculation (direct antifungal activity) of extracts of cultures grown for different periods of time was examined for suppression of *L. maculans*. Since the focus of this experiment was to assess the ability of antifungal metabolites to induce ISR or to exhibit direct antifungal activity, strains PA23 (phenazine and pyrrolnitrin) and DFE4 (lipopeptide antibiotics) were selected as representatives.

In the split inoculation assays, bacterial extract treatments—PA23 (Days 2, 3, 4, and 5) and DFE4 (Days 3 and 5) were significantly different from the control treatment, but less efficient in reducing disease compared to the local inoculation treatment (Fig. 3). In the local (SPI) inoculation assays, all bacterial extract treatments of PA23 and DFE4 (Days 1, 2, 3, 4, and 5) had significantly higher disease reduction than the control (Fig. 4). The bacterial treatments had disease ratings ranging from 0.3 to 2.8, compared to the control of 8.4 (Fig. 4). Four- and five-day-old culture extracts exhibited more antifungal activity, as seen in the low disease levels on the cotyledons.

Analysis of plant defense-related enzyme activity

Chitinase assay

Bacterial strains DF190, DFE4 and DFE16 were tested for localized induction of chitinase activity at the site of inoculation for the suppression of *L. maculans*. None of the bacterial treatments inoculated alone or together with the pathogen induced chitinase activity. For all of the treatments chitinase activity levels were less than the water control at 48, 72, 96 and 120 h after inoculation of the pathogen (Fig. 5a–c).

β-1,3 Glucanase activity

Bacterial strains DF190, DFE4 and DFE16 were tested for localized induction of β-1,3 glucanase enzyme activity at the site of inoculation. Of the three bacterial strains, only DF190 co-inoculated with the pathogen induced higher levels of β-1,3 glucanase activity at 48, 72, 96 and 120 h post-pathogen

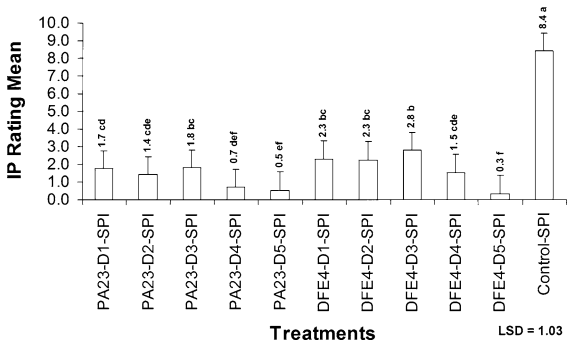

Fig. 4 Effect of single point inoculation of culture broth extracts on the suppression of *Leptosphaeria maculans* pycnidiospores on Westar cotyledons. Culture extracts of *Pseudomonas chlororaphis* strain PA23 and *Bacillus cereus* strain DFE4 were locally inoculated on the same cotyledon 24 h prior to pathogen pycnidiospores inoculation. Extracts were generated from 1, 2, 3, 4 and 5-day-old bacterial cultures. Disease was scored 10–12 days after pathogen inoculation. Analysis of variance and mean separation testing (Fisher's Least Significant Difference; $P = 0.05$) were performed ($F_{10,253} = 35.68$, $P < 0.0001$). Standard error bars are attached to the means

inoculation, as compared to the pathogen and water control (Fig. 5d). The activity peaked at 72 h and 96 h after pathogen inoculation. The enzyme activity for strain DFE4, when inoculated alone or with the pathogen, was always lower than the water control (Fig. 5e). Strain DFE16 inoculated alone had higher enzyme activity than the other treatments at 48 h and 96 h, but the bacterial treatment alone or when inoculated with the pathogen at other time intervals had enzyme activities lower than the water control (Fig. 5f).

Peroxidase activity

Bacterial strains DF190, DFE4 and DFE16 were tested for localized induction of peroxidase enzyme activity at the site of inoculation for the suppression. All of the treatments including bacteria inoculated alone, bacteria inoculated with the pathogen, and pathogen alone had peroxidase activity levels greater than the water control. For strain DF190 inoculated

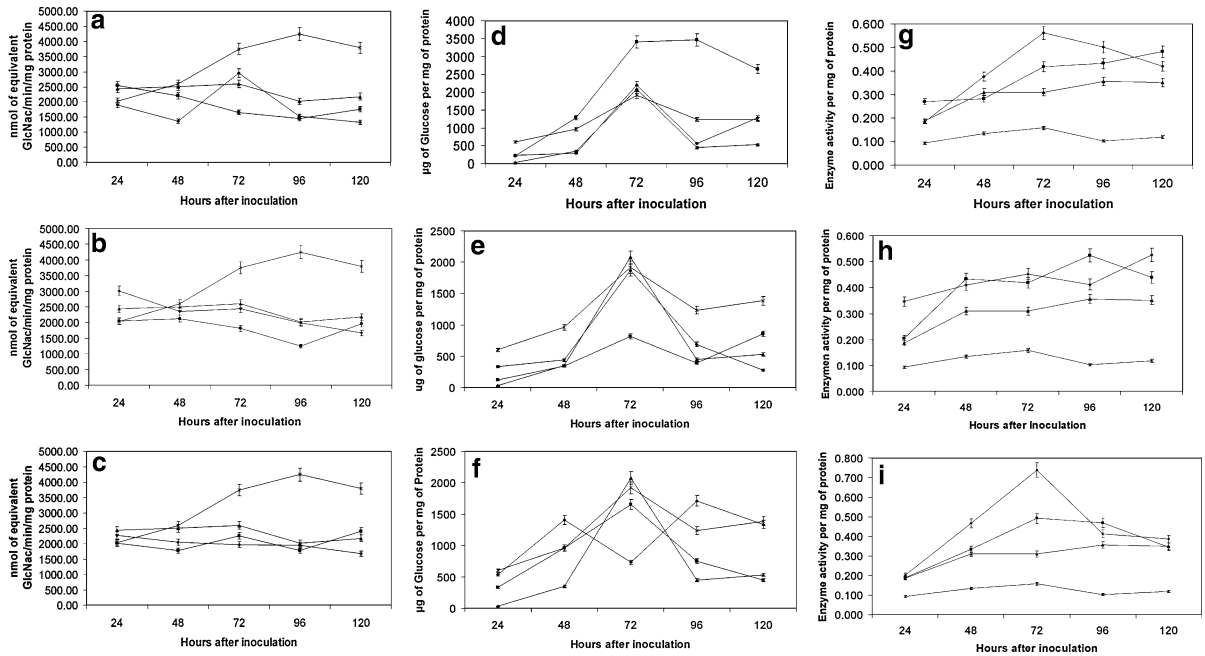

Fig. 5 Colorimetric estimation of pathogenesis-related enzyme activity upon single point inoculation of bacterial cells and *Leptosphaeria maculans* pycnidiospores. Bacterial cells were inoculated 24 h prior to pycnidiospores at the same wound site. The treatments included: bacteria inoculated alone (filled square); bacteria inoculated with the pathogen (filled diamond); pathogen inoculated alone (filled triangle); and water control (times). Chitinase activity: **a** *Pseudomonas chlororaphis* strain DF190; **b** *Bacillus cereus* strain DFE4; **c** *B. amyloliquefaciens* strain DFE16. β-1,3 Glucanase activity: **d** *P. chlororaphis* strain DF190; **e** *B. cereus* strain DFE4; **f** *B. amyloliquefaciens* strain DFE16. Peroxidase activity: **g** *P. chlororaphis* strain DF190; **h** *B. cereus* strain DFE4; **i** *B. amyloliquefaciens* strain DFE16. The vertical bars indicate the standard error of three replications

alone, peroxidase activity was higher than the other treatments at 48, 72 and 96 h. The bacterium, when inoculated with the pathogen, induced the highest enzyme activity at 120 h compared to the other treatments (Fig. 5g). When inoculated with the pathogen, the enzyme activity for strain DFE4, was higher than the other treatments at 48 h and 96 h (Fig. 5h). Strain DFE16 on its own stimulated higher peroxidase activity compared to the other treatments at 48 h and 72 h, and when co-inoculated with the pathogen, the activity was found to peak at 96 h (Fig. 5i). The overall peroxidase activity in all the treatments was low (<0.8).

Biocontrol of L. maculans using P. chlororaphis strain PA23 mutants

Tn*5* mutants of *P. chlororaphis* strain PA23 were assayed on canola cotyledons for the suppression of the pycnidiospores of *L. maculans*. The treatments with PA23-(wild type), PA23-P (pUCP23 vector control), PA23-314-G (*gacS* complemented) and PA23-63 (phenazine mutant) had significantly lower disease levels than the pathogen control, and treatments with PA23-314 (*gacS* mutant) and PA23-314-P (pUCP23 vector control) (Fig. 6a). The disease suppressive treatments of PA23 wild type (Fig. 6b), PA23-P (Fig. 6c), PA23-314-G (Fig. 6e) and PA23-63 (Fig. 6f) had IP ratings of 1.5, 1.8, 1, and 0.6, respectively. The pathogen control (Fig. 6a, g) had an IP rating of 8.6, and the mutant treatments PA23-314 (Fig. 6a, d) and PA23-314P (Fig. 6a), were not significantly different from the pathogen control.

Discussion

This study examined the ability of four antibiotic producing bacterial antagonists to suppress pycnidiospores and blackleg disease lesions on cotyledons of canola. The experimental results demonstrated direct antifungal activity to be the most dominant method of blackleg control by the four bacteria. However, low but significant disease suppression resulted from induced resistance.

The correct timing of application of the biocontrol agent, a suitable application strategy and establishment of the biocontrol agent at the target area are the critical elements that determine the successful

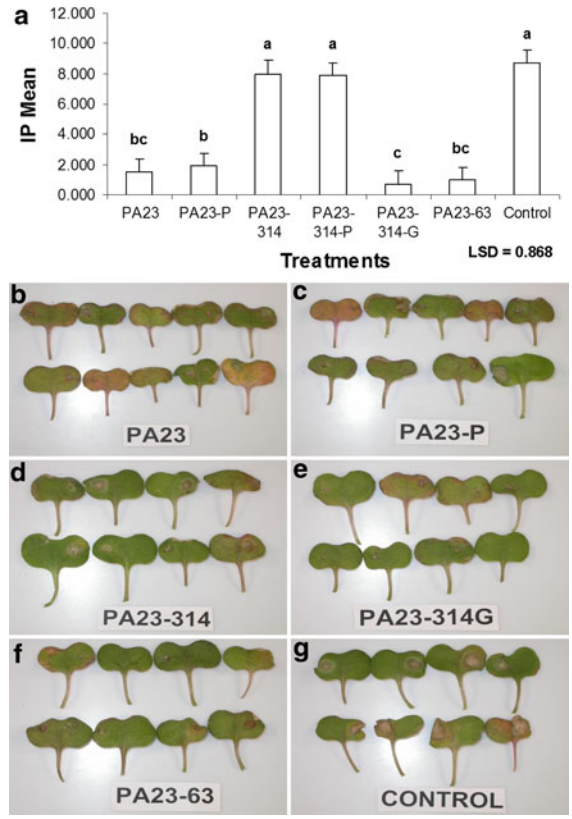

Fig. 6 Blackleg suppression by Tn*5* mutants of *Pseudomonas chlororaphis* strain PA23 on canola cv. Westar cotyledons. **a** Bacteria were inoculated 24 h prior to pycnidiospores of *Leptosphaeria maculans* on the same wound site. The bacteria that were assayed include: *P. chlororaphis* strain PA23 (wild type), PA23-P (wild type with the plasmid vector); PA23-314 (*gacS* mutant), PA23-314-P (*gacS* mutant with the plasmid vector), PA23-314-G (complemented *gacS* mutant), PA23-63 (*phzE* mutant) and CK (pathogen only control). The cotyledons were scored on a scale of 0–9, 10–12 days post pycnidiospore inoculation. Analysis of variance and mean separation testing (Fisher's Least Significant Difference; $P = 0.05$) were performed ($F_{6,245} = 247.95$, $P < 0.0001$). **b** *P. chlororaphis* strain PA23-wild type; **c** strain PA23-pUCP23; **d** strain PA23-314 (*gacS* mutant); **e** strain PA23-314–G (re-complemented with *gacS* gene from the wild type); **f** strain PA23-63 (*phzE* mutant); **g** pathogen control

management of plant pathogens through biocontrol (Baker and Cook 1974; Campbell 1989; Weller et al. 1988). In our experiment, the application of the bacteria 24 h or 48 h prior to the pathogen was an important factor in the prevention of blackleg lesions on the cotyledons, thus stressing the importance of treatment timing. Early application seems to provide the bacteria with ample time for successful colonization of the infection court, which helps to prevent

germination of pycnidiospores and thus the penetration and establishment of the pathogen in the host. This conclusion is further supported by our results showing that co-inoculation or post-pycnidiospore inoculation of the bacteria did not suppress the disease in the cotyledon. In this case, the pathogen pycnidiospores may have germinated and penetrated the wound area before the establishment and colonization by the bacteria. Earlier studies by Bull et al. (1991) on root colonization by *P. fluorescens* in the suppression of *Gaeumannomyces graminis* var. *tritici*, and Parke (1990) on the successful colonization of the pea spermosphere in the prevention of *Pythium* infection, stress the importance of colonization of the biocontrol agent at the target site as a prerequisite for suppression of plant pathogens.

In our study, we inoculated the bacteria and the pathogen on two separate cotyledons to test for the induction of systemic resistance by the bacteria. Similar phyllosphere inoculation studies have been carried out for the induction of ISR with *B. mycoides* strain Bac J for the control of *Cercospora* leaf spot in sugar beet (Bargabus et al. 2002). Other examples include, protection of leaves from bacterial pustule, caused by *Xanthomonas axonopodis* pv. Glycines (Xag) through soybean seed inoculation with *Bacillus amyloliquefaciens* KPS46 (Buensanteai et al. 2009); and *Pseudomonas putida* WCS358r and *P. fluorescens* WCS374r for the control of bacterial wilt caused by *Ralstonia solanacearum* in *Eucalyptus urophylla* (Ran et al. 2005). The fluorescent siderophore pseudobactin, LPS, and the flagella of *P. putida* WCS358 (Bakker et al. 2003; Meziane et al. 2005) have been demonstrated as inducers of ISR.

Other than siderophores, LPS and flagella as bacterial determinants of ISR, antifungal antibiotics produced by bacteria have also been shown to induce ISR. Examples of ISR-inducing antibiotics include pyocyanin (Audenaert et al. 2002), 2,4-diacetylphloroglucinol (Iavicoli et al. 2003), and pyrrolnitrin (Pang et al. 2009). Lipopeptide antibiotics produced by *Bacillus* spp., such as fengycin (Ongena et al. 2005) and surfactin (Ongena et al. 2007) also induce systemic resistance. Jourdan et al. (2009) further investigated the molecular events involved in the induction of ISR in tobacco cells following treatment with surfactin. Surfactin induced defense-related early events such as extracellular medium alkalinization coupled with ion fluxes and reactive oxygen species production. It stimulated the defense enzymes phenylalanine ammonialyase and lipoxygenase and modified the pattern of phenolics produced by the elicited cells. Surfactin also activated Ca^{2+} influx and dynamic changes in protein phosphorylation but did not cause any marked phytotoxicity or adverse effect on the integrity and growth potential of the treated tobacco cells (Jourdan et al. 2009). We have shown previously that *P. chlororaphis* strains PA23 and DF190 produce phenazines (phenazine-1-carboxylic acid, 2-hydroxyphenazine) and pyrrolnitrin (Zhang et al. 2006; Ramarathnam 2007), and *B. cereus* strain DFE4 and *B. amyloliquefaciens* strain DFE16 produce the lipopeptide antibiotics iturin A, bacillomycin D and surfactin (Ramarathnam 2007). Therefore, strains PA23 and DFE4 were selected as representative producers of each set of antibiotics. The split inoculation of the extract (for induction of ISR) showed a small but significant reduction in disease severity via a systemic response. The local (SPI) inoculation of the extract (for direct antagonism) showed significantly higher reduction of disease levels, which is also consistent with the SPI of the bacterial cells, establishing a more important role for the antifungal metabolites present in the culture extracts for the direct suppression of *L. maculans*. To further ascertain the mechanism involved in the localized disease suppression, the activity of PR enzymes was studied. Strain DF190 increased β-1, 3 glucanase production and peroxidase activity when inoculated with the pathogen. However, the potential for these bacteria to exhibit direct antifungal activity at the site of infection seems more prominent. Direct antifungal activity could result from the production of antibiotics or extracellular lytic enzymes. *L. maculans* can cause leaf infection only through penetration of natural openings or wounds, which act as sites of infection (Hammond et al. 1985). Wounds and natural openings of the leaf are nutrient rich microenvironments, which favor the production of bacterial secondary metabolites, like antibiotics (Thomashow et al. 1997). The localized inhibition of pycnidiospores by the bacteria is enabled by successful colonization of the infection site, which in turn most probably acts as a suitable delivery system for the antifungal metabolites. This phenomenon was observed in the suppression of *Fusarium oxysporum* f. sp. *radicis-lycopersici* through effective root colonization and production of phenazine-1-carboxamide

by *P. chlororaphis* PCL1391 (Chin-A-Woeng et al. 2000).

The GacS/GacA system controls the expression of genes required for the synthesis of secondary metabolites, such as antibiotics in many plant-associated fluorescent *Pseudomonas* species (Heeb and Haas 2001). In *P. chlororaphis* strain PA23, a *gacS* mutation leads to a deficiency in secondary metabolite production and an inability to control *S. sclerotiorum* in canola (Poritsanos et al. 2006). Similar results were observed in our study. A *gacS* mutation led to loss of *L. maculans* biocontrol activity by the mutant PA23-314. As expected, the biocontrol activity was restored when the *gacS* gene was added in trans to PA23-314. The complemented mutant PA23-314 *gacS* exhibited enhanced antifungal activity and this is likely due to a gene dosage effect. The phenazine mutant PA23-63 exhibited antifungal and biocontrol activity similar to that of the wild type. Mutant PA23-63 produces pyrrolnitrin at elevated levels compared to the wild type (Selin et al. 2010). The biocontrol activity mediated by mutant PA23-63, despite its lack of phenazine production, establishes that phenazines are not essential for biocontrol of *L. maculans*. It is possible that even in the wild type, which produces both phenazine and pyrrolnitrin, no synergistic activity between phenazine and pyrrolnitrin exist. We postulate that pyrrolnitrin and extracellular lytic enzymes could be responsible for the antifungal activity of *P. chlororaphis* strain PA23 towards *L. maculans* while there is a small but significant disease suppression observed through induced resistance mechanisms.

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References

Audenaert K, Pattery T, Cornelis P, Hofte M (2002) Induction of systemic resistance to *Botrytis cinerea* in tomato by *Pseudomonas aeruginosa* 7NSK2: role of salicylic acid, pyochelin, and pyocyanin. Mol Plant Microbe Interact 15:1147–1156

Baker KF, Cook RJ (eds) (1974) Biological control of plant pathogens. W.H. Freeman and Company, San Francisco, 433 pp

Bakker PAHM, Ran LX, Pieterse CMJ, van Loon LC (2003) Understanding the involvement of rhizobacteria-mediated induction of systemic resistance in biocontrol of plant diseases. Can J Plant Pathol 25:5–9

Bargabus RL, Zidack NK, Sherwood JW, Jacobsen BJ (2002) Characterization of systemic resistance in sugar beet elicited by a non-pathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent. Physiol Mol Plant Pathol 61:289–298

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

Buensanteai N, Yue GY, Prathuangwong S (2009) Priming, signaling, and protein production associated with induced resistance by *Bacillus amyloliquefaciens* KPS46. World J Microbiol Biotechnol 25:1275–1286

Bull CT, Welle DM, Thomashow LS (1991) Relationship between root colonization and suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* strain 2-79. Phytopathology 81:954–959

Campbell R (1989) Biological control of microbial plant pathogens. Cambridge University Press, Cambridge

Chin-A-Woeng TFC, Bloemberg GV, Mulders IHM, Dekkers LC, Lugtenberg BJJ (2000) Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. Mol Plant Microbe Interact 12:1340–1345

de Boer M, van der Sluis I, van Loon LC, Bakker PAHM (1999) Combining fluorescent *Pseudomonas* spp. strains to enhance suppression of fusarium wilt of radish. Eur J Plant Pathol 105:201–210

De Vleesschauwer D, Djavaheri M, Bakker PAHM, Hofte M (2007) *Pseudomonas fluorescens* WCS374r-induced systemic resistance in rice against *Magnaporthe oryzae* is based on pseudobactin-mediated priming for a salicylic acid-repressible multifaceted defense response. Plant Physiol 148:1996–2012

Fernando WGD, Nakkeeran S, Zhang Y, Savchuk S (2007) Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary by *Pseudomonas* and *Bacillus* species on canola petals. Crop Prot 26:100–107

Hammerschmidt R, Nuckles EM, Kuc J (1982) Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotricum lagenarium*. Physiol Mol Pathol 20:73–82

Hammond KE, Lewis BG, Musa TM (1985) A systemic pathway in the infection of oilseed rape plants by *Leptosphaeria maculans*. Plant Pathol 34:557–565

Heeb S, Haas D (2001) Regulatory roles of the GacS/GacA two-component system in plant-associated and other gram-negative bacteria. Mol Plant Microbe Interact 14:1351–1363

Iavicoli A, Boutet E, Buchala A, Metraux JP (2003) Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. Mol Plant Microbe Interact 16:851–858

Jourdan E, Henry G, Duby F, Dommes J, Barthélemy JP, Thonart P, Ongena M (2009) Insights into the defense-related events occurring in plant cells following perception of surfactin-type lipopeptide from *Bacillus subtilis*. Mol Plant Microbe Interact 22:456–468

McCleary BV, Shameer I (1987) Assay of malt beta-glucanase using azo-barley glucan—an improved precipitant. J Inst Brew 93:87–90

Meziane H, Sluis IVD, van Loon LC, Hofte M, Bakker PAHM (2005) Determinants of *Pseudomonas putida* WCS358 involved in inducing systemic resistance in plants. Mol Plant Pathol 6:177–185

Olivain C, Alabouvette C, Steinberg C (2004) Production of a mixed inoculum of *Fusarium oxysporum* Fo47 and *Pseudomonas fluorescens* C7 to control *Fusarium* diseases. Biocontrol Sci Technol 14:227–238

Ongena M, Jacques P, Touré Y, Destain J, Jabrane A, Thonart P (2005) Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of *Bacillus subtilis*. Appl Microbiol Biotechnol 69:29–38

Ongena M, Jourdan E, Adam A, Paquot M, Bran A, Joris B, Arpigny JL, Thonart P (2007) Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environ Microbiol 9:1084–1090

Pang Y, Liu X, Ma Y, Chernin L, Berg G, Gao K (2009) Induction of systemic resistance, root colonization and biocontrol activities of the rhizospheric strain of *Serratia plymuthica* are dependent on N-acyl homoserine lactones. Eur J Plant Pathol 124:261–268

Parke JL (1990) Population dynamics of *Pseudomonas cepacia* in the pea spermosphere in relation to biocontrol of *Pythium*. Phytopathology 80:1307–1311

Pieterse CMJ, van Pelt JA, van Wees SCC, Ton J, Kloosterzie KML, Keurentjes JJB, Verhagen VWM, Knoester M, Sluis IVD, Bakker PAHM, van Loon LC (2001) Rhizobacteria-mediated induced systemic resistance: triggering, signaling and expression. Eur J Plant Pathol 107:51–61

Poritsanos NJ (2005) Molecular mechanisms involved in the secondary metabolite production and biocontrol of *Pseudomonas chlororaphis* PA23. Masters Thesis Dissertation, University of Manitoba

Poritsanos N, Selin C, Fernando WGD, Nakkeeran S, de Kievit TR (2006) A GacS deficiency does not affect *Pseudomonas chlororaphis* PA23 fitness when growing on canola, in aged batch culture or as a biofilm. Can J Microbiol 52:1177–1188

Ramarathnam R (2007) Mechanisms of phyllosphere biological control of *Leptosphaeria maculans*, the blackleg pathogen of canola, using antagonistic bacteria. PhD Thesis, University of Manitoba

Ramarathnam R, Fernando WGD (2006) Preliminary phenotypic and molecular screening for potential bacterial biocontrol agents of *Leptosphaeria maculans*, the blackleg pathogen of canola. Biocontrol Sci Technol 16:567–582

Ran LX, Li ZN, Wu GJ, van Loon LC, Bakker PAHM (2005) Induction of systemic resistance against bacterial wilt in *Eucalyptus urophylla* by fluorescent *Pseudomonas* spp. Eur J Plant Pathol 113:59–70

Selin C, Habibian R, Poritsanos N, Athukorala SNP, Fernando D, De Kievit TR (2010) Phenazines are not essential for *Pseudomonas chlororaphis* PA23 biocontrol of *Sclerotinia sclerotiorum*, but do play a role in biofilm formation. FEMS Microbiol Ecol 71:73–83

Thomashow LS, Bonsall RF, Weller DM (1997) Antibiotic production by soil and rhizosphere microbes in situ. In: Hurst CJ, Knusden GR, McInerney MJ, Stetzenbach LD, Walter MD (eds) Manual of environmental microbiology. ASM Press, Washington, DC, pp 493–499

Tran H, Ficke A, Asiimwe T, Höfte M, Raaijmakers JM (2007) Role of the cyclic lipopeptide massetolide A in biological control of *Phytophthora infestans* and in colonization of tomato plants by *Pseudomonas fluorescens*. New Phytol 175:731–742

Weller DM, Howie WJ, Cook RJ (1988) Relationship between in vitro inhibition of *Gaeumannomyces graminis* var *tritici* and suppression of take-all of wheat by fluorescent pseudomonads. Phytopathology 78:1094–1100

Zhang Y, Fernando WGD, de Kievit T, Berry C, Daayf F, Paulitz T (2006) Detection of antibiotic-related genes from bacterial biocontrol agents with polymerase chain reaction. Can J Microbiol 52:476–481