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RESEARCH ARTICLE

The role of volatile and non-volatile antibiotics produced by *Pseudomonas chlororaphis* strain PA23 in its root colonization and control of *Sclerotinia sclerotiorum*

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Pseudomonas chlororaphis strain PA23 has demonstrated excellent biocontrol in the canola phyllosphere. This bacterium produces the non-volatile antibiotics phenazine and pyrrolnitrin as well as the volatile antibiotics nonanal, benzothiazole and 2-ethyl-1-hexanol. *In vitro* experiments were conducted to study the effects of different mutations on the production of these three organic volatile antibiotics by PA23. *In planta* experiments in the greenhouse investigated the role of the non-volatile antibiotics on root colonization and biocontrol ability of PA23 against *Sclerotinia sclerotiorum* on sunflower. Analysis of phenazine- and pyrrolnitrin-deficient *Tn* mutants of PA23 revealed no differences in production of the three volatile antibiotics. On all sampling dates, PA23 applied alone or in combination with the mutants showed significantly higher ($P = 0.05$) root bacterial number and Sclerotinia wilt suppression ($P = 0.05$). Decline of the bacterial population seemed to be inversely proportional to/or negatively correlated with the number of antibiotics produced by PA23 but the relative importance of phenazine or pyrrolnitrin on root colonization and/or wilt suppression was not clear. In several cases, the strains producing at least one antibiotic maintained relatively higher bacterial numbers than non-producing strains. However, by 6 weeks after sowing, there was a rapid and significant ($P = 0.05$) increase in the proportion of introduced bacteria capable of producing at least one antibiotic over the total bacterial population. Furthermore, combining certain mutants with PA23 reduced the root colonization and biocontrol ability of PA23. Strain PA23-314 (*gacS* mutant) showed competitive colonization in comparison to the other mutants for most sampling dates.

Keywords: *Pseudomonas chlororaphis* PA23; volatile and non-volatile antibiotics; root colonization

Introduction

Pseudomonas chlororaphis strain PA23, a bacterium originally isolated from the root tips of soybean plants (Savchuk 2002), was shown to be capable of protecting canola from the pathogenic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary both in greenhouse and field studies (Savchuk and Fernando 2004; Zhang 2004; Fernando, Nakkeeran, Zhang and Savchuk 2007). *P. chlororaphis* biocontrol is thought to result from

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production of non-volatile organic compounds, such as phenazine-1-carboxylic acid (PCA), 2-hydroxyphenazine and pyrrolnitrin (Zhang et al. 2006) together with several metabolites including protease, lipase, HCN, and siderophores (Poritsanos, Selin, Fernando, Nakkeeran and de Kievit 2006). In addition, PA23 produces three volatile organic compounds, namely nonanal, benzothiazole and 2-ethyl–1-hexanol, which showed 100% inhibition of mycelial and sclerotial germination of *S. sclerotiorum* (Fernando, Ramarathnam, Krishnamoorthy and Savchuk 2005). The success of strain PA23 as a biocontrol agent (BCA) against *Sclerotinia* on canola suggested that it might serve as a potential BCA against Sclerotinia infection of sunflower.

In sunflower *S. sclerotiorum* causes three main diseases; head rot, mid-stalk rot, and wilt. In terms of yield losses, Sclerotinia wilt is considered the most important sunflower disease in North America (Gulya 1985, 1996). In Manitoba, wilt is reported every year and is considered to be one of the limiting factors affecting sunflower production (Putt 1958; Hoes and Huang 1976; Rashid and Seiler 2005). Early infection in the plant may cause 100% yield loss by killing the plant before flowering, whereas later infections may result in 80% yield loss (Rashid and Seiler 2005). Consequently control of this disease is a significant issue in Manitoba.

Biological control is the preferred means of disease management because of economic and environmental problems associated with chemical and culturing methods for disease control. Colonization of the plant root and the rhizosphere by the BCA is one way to prevent establishment of plant pathogens in root-borne diseases. Successful colonization of the introduced BCA requires adaptation to the new environment and a selective advantage over the indigenous microflora already present in the rhizosphere (Mazzola, Cook, Thomashow, Weller and Pierson 1992). Antibiotics produced by the BCA have been found to play an important role in colonization (Bruehl, Millar and Cunfer 1969; Atlas and Bartha 1987; Mazzola et al. 1992), although this point is contentious (Gottlieb 1976; Williams 1982; Williams and Vickers 1986). Antibiotic production may benefit a particular microorganism by inhibiting rhizosphere competitors and facilitating its own colonization. To develop successful strategies for field application of an antibiotic-producing BCA, studies on its pattern of root colonization and the involvement of antibiotics in this process are required.

The objectives of this study were 1) to determine how various mutations in antibiotic-related genes affect organic volatile antibiotics produced by PA23; 2) to determine whether the non-volatile antibiotics phenazine and pyrrolnitrin are important for PA23 root colonization and Sclerotinia wilt control by using phenazine and/or pyrrolnitrin deficient mutants of PA23.

Materials and methods

Bacterial strains, fungal strain and culture conditions

Cultures of all bacterial strains were started by streaking the bacteria onto Luria Bertani agar (LBA) (Difco Laboratories, Detroit, MI, USA) amended with appropriate antibiotics (Table 1) from stock cultures maintained in Luria Bertani broth (LBB) containing 20% glycerol at −80°C. The fungal strain *S. sclerotiorum* SS33 (obtained from Linda Khon's lab) was used in all the experiments. Fresh cultures were started from surface-sterilized sclerotia placed on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI, USA) incubated at room temperature.

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Table 1. List of bacterial strains used in the study, their characteristics and type and amount of antibiotics added to the medium to grow each strain.

		Characteristics			
Strain name	Mutation/ modification	Non-volatile antibiotics	HCN	Antibiotic selection (μg/mL)	Reference/source
PA23	Wild type	(+) Pyrrolnitrin (+) Phenazine	+	Rifampicin: 25	
PA23-314	*gacS* – global regulator	(–) Pyrrolnitrin (–) Phenazine	–	Tetracycline: 15	Poritsanos et al. (2006)
PA23-314 *gacS*	*gacS* complemented strain PA23-314	(+) Pyrrolnitrin (+) Phenazine	+	Gentamicin: 20	Poritsanos et al. (2006)
PA23-63	*phzE*	(+) Pyrrolnitrin (–) Phenazine	+	Tetracycline: 15	Selin et al. (2010)
PA23-754	*phzC*	(+) Pyrrolnitrin (–) Phenazine	+	Tetracycline: 15	Poritsanos (2005)
PA23-443	*ptrA* – lysR-type transcriptional regulator	(–) Pyrrolnitrin (–) Phenazine	–	Tetracycline: 15	Poritsanos (2005)
PA23-443 *ptrA*	*ptrA* complemented strain PA23-443	(+) Pyrrolnitrin (+) Phenazine	+	Gentamicin: 20	Poritsanos (2005)
PA23-1	*prnBC*	(–) Pyrrolnitrin (+) Phenazine	+	Gentamicin: 20	Selin et al. (2010)
PA23-63-1	*phzE*/*prnBC*	(–) Pyrrolnitrin (–) Phenazine	+	Gentamicin 20/tetracycline 15	Selin et al. (2010)

Effect of bacterial volatiles on mycelial growth of S. sclerotiorum *(divided plate method)*

Wild-type strain PA23 and its derivatives (PA23-314, PA23-314 *gacS*, PA23-63, PA23-754, PA23-443, and PA23-443 *ptrA*) were streaked separately onto half of a divided plate containing tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI, USA) amended with appropriate antibiotics. The plate was immediately wrapped with Parafilm® (Pechinery Plastic Packaging, Menasha, WI, USA) to trap the bacterial volatiles and incubated for 5 days at 28°C. Following the incubation period, a 5 mm mycelial plug of *S. sclerotiorum* was placed on the other half of the divided plate containing PDA and the plates were resealed. Measurements of radial mycelial growth were taken at 24 h intervals post-inoculation for 5 days. The percentage inhibition (PI) was calculated when the mycelia reached the periphery just opposite to the bacterial culture in the control plate using the formula $[(T\text{-}C)/C] \times 100$, where T = radial mycelial growth in treatment and C = radial mycelial growth in the control. There were ten replicates for each treatment and the experiment was performed three times.

Effect of bacterial volatiles on sclerotial germination of S. sclerotiorum

The bacterial treatments and the methods are the same as those described in the previous experiment with the following exception. After the 5-day incubation period, sclerotia that had been cut into two halves (approximately 2 mm in diameter) were placed on the other side of the divided plate containing PDA. The sclerotia were placed on a PDA plate to start germination prior to placing them in the divided plate to confirm their viability. The percentage inhibition (PI) was calculated when the mycelia reached the periphery just opposite to the bacterial culture in the control plate using the formula $[(T\text{-}C)/C] \times 100$, where T = radial mycelial growth in treatment and C = radial mycelial growth in the control. There were ten replicates for each treatment and the experiment was performed three times.

Production of ascospores

Ascospores used in this study were produced as follows: sclerotia that had been grown on PDA plates at 15°C were incubated at 4°C for two weeks and then surface sterilized with a 10% (v/v) solution of NaOCl in distilled water (store brand bleach having an initial concentration of 4.0%) prior to placement on the vermiculite in glass Petri plates. Stipes appeared after 2 to 4 months, at which time sclerotia were transferred to 10 mm diameter Petri dishes containing 1.0% water agar. Ascospores were harvested from apothecia after approximately 2–5 weeks using vacuum filtration onto a Millipore© membrane filter (type GS, 47 μm) placed in a 150 ml bottle top filter (0.22 μm CN) with 45-mm neck (Corning®, Corning, NY, USA). They were stored in a desiccator at 4°C until use. Excellent spore viability has been reported after 24 months using this protocol (Hunter, Steadman and Cigna 1982).

Spores were recovered by adding Tween 20 (polyoxyethylene (20) sorbitan monolaurate, Mallinckrodt OR®, Paris, KY, USA) as a surfactant to the spore suspension and the mixture was vigorously vortexed for 1 min. to break up clumps of spores prior to enumeration using a haemocytometer.

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Effect of bacterial volatiles on ascospore germination of S. sclerotiorum

A 20-μL aliquot of ascospore suspension (5×10^4 spores/ml of 0.1 M phosphate buffer pH 7.0) was placed on a cavity slide. The slide was then placed inside the bottom dish of a sterile Petri plate. Another bottom dish containing a 24-h culture of each of the bacterial strains listed in Table 1 (except PA23-1 and PA23-63-1) growing on TSA was inverted on the dish containing the cavity slide and the two dishes were sealed together using Parafilm. After 24 and 48 h incubation at room temperature, the slides were observed for spore germination under a microscope. Ten microscopic fields were selected (10×45x) and in each field the number of germinated spores was counted. The percentage inhibition of spore germination was calculated compared to the control. There were four replicates for each bacterium, and the experiment was performed twice.

Collection and analysis of volatile organic compounds

Headspace volatiles produced by each bacterium were collected using a setup described by DeMilo, Lee, Moreno and Martinez (1996) with slight modifications. Bacteria were grown in either 100 ml of tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA) or M9 minimal medium (per litre: 16 g $NaHPO_4$-$7H_2O$, 3.75 g KH_2PO_4, 0.625 g NaCl, 1.25 g NH_4Cl, 0.24 g 1M $MgSO_4$, 0.01 g 1M $CaCl_2$, and 20 ml of 20% glucose as the carbon source). After 5 days, the headspace volatiles were collected into a volatile trap (7cm length and 0.4 cm diameter) containing 150 mg of activated charcoal (Darco®. 20-40 mesh, Aldrich, Milwaukee, WI, USA) by using a stream of dry nitrogen (300 ml/min) for 24-48 h. Volatiles in the trap were extracted into glass vials with 500 μl of methyl chloride and analyzed through gas chromatography and mass spectrometry as described by Fernando et al. (2005), except that the column temperature was programmed from 35 to 200°C at 10°C/min ramp rate.

Root colonization study

This experiment was designed to understand the relative importance of phenazine and pyrrolnitrin towards the root colonization ability of PA23. The experiment was carried out in a standard non-sterile soil mix: 2 parts soil, 1 part sand, 1 part peat and (granular fertilizer 11-53-0) using sunflower (*Helianthus annuus* L.) cultivar IS6111. Ten pots (pot diameter 4'', height 6'') were used per treatment and the experiment was performed thrice. The experiment included the following treatments: (i) control (no bacterial treatment for seeds); (ii) PA23; (iii) PA23-314; (iv) PA23-314 *gacS*; (v) PA23-63 (*phz*$^-$); (vi) PA23-1 (*prn*$^-$); (vii) PA23-63-1 (*phz*$^-$, *prn*$^-$); (viii) PA23 + PA23-314; (ix) PA23 + PA23-314 *gacS*; (x) PA23 + PA23-63; (xi) PA23 + PA23-1; (xii) PA23 + PA23-63-1. Sunflower seeds were surface sterilized with diluted NaOCl (4:1; Chlorox) for 3 min followed by three washings with sterile distilled water. Seeds were air dried overnight under a sterile air flow. Seeds were then soaked in bacterial suspensions (10^8 CFU/ml) in 1% methylcellulose (Sigma®, Sigma Chemical Co., St. Louis, MO, USA) in 0.1 M sodium phosphate buffer (pH 7.0) for 1 h, and air dried overnight under a sterile air flow. The initial bacterial count on seeds was determined using a standard dilution plate method (10 seeds suspended in 100 ml sterile sodium phosphate buffer pH 7.0). One seed was planted per pot to a

depth of 1.5 cm and pots were placed in the growth room for 10 weeks at 21°C and 19°C (day and night temperatures with 16 h photoperiod) and watered daily. Root bacterial counts were determined every two weeks (at the end of the 2nd, 4th and 6th week after sowing) for 6 weeks starting from the 14th day after planting as follows. Two seedlings were collected randomly from each treatment. The seedlings were shaken gently to remove soil so that only the most tightly adhering rhizosphere soil remained. Roots from both seedlings were pooled, and standard dilution plating was performed with 0.1 g of root (at seedling stages) and 1g of root (at mature stages) by suspending the roots in 10 ml of sterile distilled water. The root suspension was sonicated for 60 s followed by vortexing for 5 s to suspend the bacteria that were colonizing the root. For each treatment dilutions were plated on nutrient agar (Difco Laboratories, Detroit, MI, USA) amended with appropriate antibiotics to determine the number of introduced bacteria, and then on nutrient agar without antibiotics to obtain the total bacterial count on roots.

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Early wilt disease incidence

The conditions for growing plants in this experiment were the same as in the root colonization experiment and the two experiments were carried out simultaneously. The treatments were (i) negative control (no bacterial or *Sclerotinia* treatment); (ii) positive control (no bacterial treatment but with *Sclerotinia*) (iii) bacterial strain PA23; (iv) PA23-314; (v) PA23-314 *gacS*; (vi) PA23-63 (*phz*$^-$); (vii) PA23-1 (*prn*$^-$); (viii) PA23-63-1 (*phz*$^-$, *prn*$^-$); (ix) PA23 + PA23-314; (x) PA23 + PA23-314 *gacS*; (xi) PA23 + PA23-63; (xii) PA23 + PA23-1; (xiii) PA23 + PA23-63-1; (xiv) fungicide (Ronilan: active ingredient vinclozolin) all inoculated with *Sclerotinia*. The fungicide treatment was used as a comparison to BCA. For the fungicide treatment, the seeds were mixed with slightly wetted fungicide (4g/1 kg of seeds) and air-dried overnight. To inoculate *Sclerotinia*, the pots were filled ¾ full with soil mix and each pot was sprinkled with 1g of Sclerotinia-infected pearl millet seeds to mimic sclerotia prior to seeding. One seed was placed on the millet sprinkled soil surface and was covered with soil mix to a thickness of 1.5 cm. Each treatment was composed of 10 pots. Pots were placed in a growth room for 3 weeks and watered daily. The percent emergence of the seedlings was determined at 14 days after planting. The experiment was performed thrice.

Data analysis

Data was analyzed using analysis of variance (ANOVA) and Fisher's Least Significant Difference test at $P = 0.05$ using the Analyst procedure of SAS, Version 8.1 (SAS Institute, Carry, NC, USA).

Results

Effects of bacterial volatiles on mycelial growth of S. sclerotiorum

Mutants PA23-314 *gacS*, PA23-63, PA23-754, and PA23-443 *ptrA* significantly ($P = 0.05$) inhibited mycelial growth of *S. sclerotiorum* in comparison with the control. PA23-754 exhibited the greatest inhibition at 25.6% (Figures 1 and 2). The

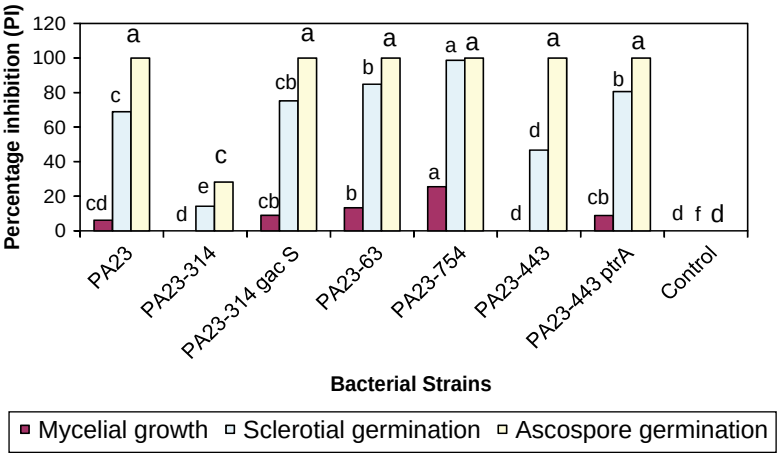

Figure 1. (Color online) Percentage inhibition (PI) shown by each bacterial strain against mycelial growth, sclerotial germination and ascospore germination. Mycelial plugs and half cut partially germinated sclerotia were inoculated onto the other half of the divided Petri plate containing PDA after 5 days of incubation of each bacterium at 28°C. Ascospores of *Sclerotinia sclerotiorum* were introduced into the experimental set up after 24 h of incubation of each bacterium at 28°C. The graph shows the PI of mycelial growth and sclerotial germination 72 h post inoculation of *S. sclerotiorum* and PI of ascospore germination 48 h post inoculation of ascospores of *S. sclerotiorum.* Series of bars sharing a single color and are followed by the same letter are not significantly different, $P = 0.05$, using the Fisher's least significant difference test. LSD: ■ 6.498 □ 10.767 □ 2.71.

mutants PA23-314 and PA23-443 did not show any significant inhibitory effect. These results are comparable with those obtained using radial diffusion assays, which were carried out to test the effect of non-volatile antibiotics produced by PA23 and its mutant derivatives (Poritsanos 2005). Control plates showed normal mycelial growth.

Effect of bacterial volatiles on sclerotial germination of S. sclerotiorum

Bacterial mutants PA23-314 *gacS*, PA23-63, PA23-754, and PA23-443 *ptrA* showed significant ($P = 0.05$) inhibition of sclerotial germination in comparison with the control 72 h post inoculation of sclerotia. As observed for the mycelial growth inhibition assays, PA23-754 showed the highest percentage inhibition of sclerotial germination (Figures 1 and 2). In contrast, strains PA23-314 and PA23-443 showed considerable inhibition of sclerotial germination, which was not observed for mycelial inhibition. Inhibition shown by PA23-443 was greater than that of PA23-314 (Figure 1). In general, all bacterial strains exhibited better inhibition of *S. sclerotiorum* sclerotial germination than mycelial growth.

Effect of bacterial volatiles on S. sclerotiorum *ascospore germination*

Except for mutant strain PA23-314, all other mutants as well as the wild-type strain PA23 completely (100%) inhibited ascospore germination of *S. sclerotiorum* after 48 h of incubation at room temperature (Figure 1).

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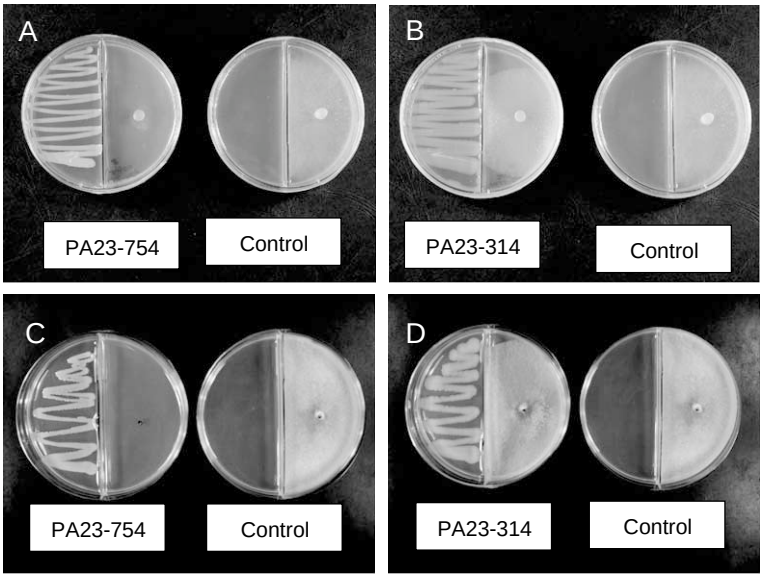

Figure 2. Antifungal-volatile activity in divided plates 72 h post inoculation of mycelial plugs and sclerotia. (A) Mycelial growth inhibition in the presence of PA23-754. (B) Normal mycelial growth in the presence of PA23-314. (C) Sclerotial germination inhibition in the presence of PA23-754. (D) Normal sclerotial germination in the presence of PA23-314. Mycelial plugs and half cut partially germinated sclerotia were inoculated on to other half of the divided Petri plate containing PDA after 5 days of incubation of each bacterium at 28°C.

GC-MS analysis of volatiles

The chromatograms obtained from each strain revealed that all of the mutants produced the same three organic volatile compounds, nonanal, benzothiazole, and 2-ethyl-1-hexanol, as the wild-type strain regardless of the medium used. Figure 3 shows representative chromatograms from PA23, PA23-314 and the complemented strain PA23-314 *gacS*.

Introduced bacterial count

Results of each sampling date were analyzed separately. The initial bacterial density on seeds was not significantly different ($P = 0.05$) among bacterial strains and ranged from log 5.6 to log 4.46/g of root (data not shown).

Two, four, and six weeks after sowing, the bacterial numbers on roots were significantly different ($P = 0.05$) from each other (Table 2). In general, the results among different sampling dates were quite variable; however, there were some significant trends that could be observed. The PA23 wild-type bacterial strain showed relatively higher root colonization compared to other strains. For most of the sampling dates, strain PA23-314 had high levels of root colonization, which on occasion was equal to that of the wild-type strain and complemented strain PA23-314 *gacS* (Table 2). The degree of root colonization by other mutant strains is not consistent over the sampling dates. When strain PA23-63-1 was applied to the root with strain PA23, PA23 showed the lowest level of root colonization (Table 2). Even

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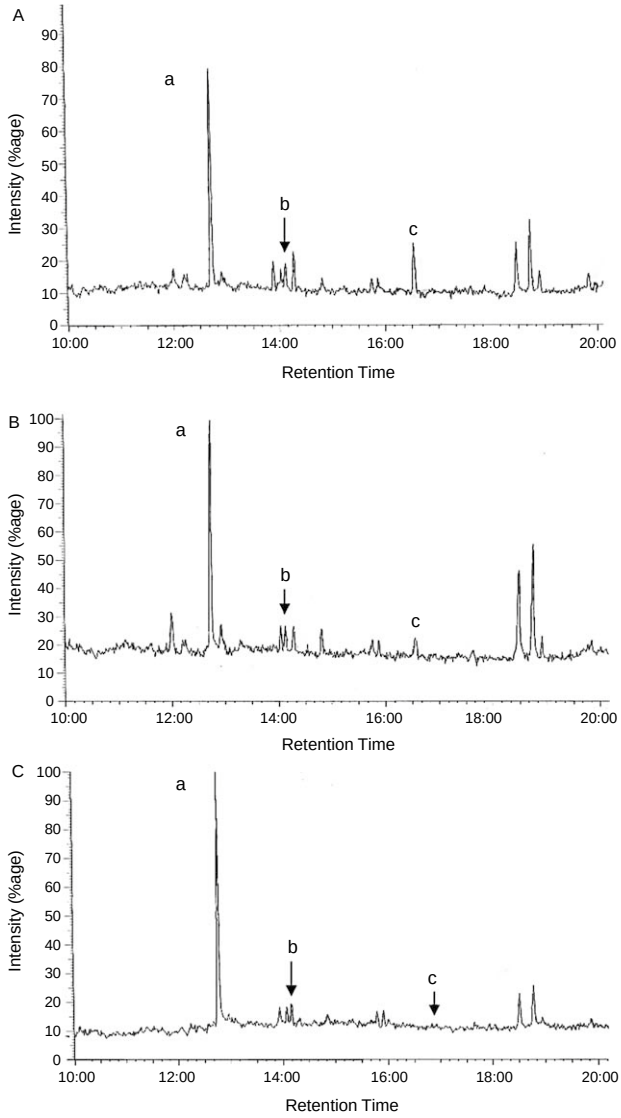

Figure 3. Chromatograms of volatiles collected from (A) PA23 and (B) PA23-314 (C) PA23-314 *gacS* grown in M9 medium; (a) 2-ethyl-1-hexanol, (b) nonanal, (c) benzothiozole. Volatiles were trapped into activated charcoal and extracted with methyl chloride and subjected to GC–MS analysis. *X*-axis represents the retention time, *Y*-axis represents the peak intensity (percentage).

though PA23-314 *gacS* and PA23-63 produce antibiotics and were therefore expected to retain high bacterial numbers, the populations decreased markedly from the second to the sixth week after sowing. For most treatments, the bacterial counts on the root increased from initial colonization up to 2 weeks and started to decrease by week 4, having very low bacterial counts by week 6 compared to the initial counts on

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Table 2. Number of colony forming units (log CFU) of introduced *Pseudomonas chlororaphis* PA23 and its various mutants alone or in combinations, re-isolated from 1 g of seminal roots of sunflower, over a period of 6 weeks after sowing.

Strain	2 weeks	4 weeks	6 weeks
PA23	5.59^{ab}	5.91^{a}	5.27^{ab}
PA23-314	5.57^{ab}	5.50^{c}	4.42^{bcde}
PA23-314 *gacS*	4.83^{ab}	5.11^{d}	3.51^{efgh}
PA23-63	2.39^{b}	4.36^{e}	2.74^{ghi}
PA23-1	5.30^{ab}	3.44^{h}	2.54^{hi}
PA23-63-1	5.55^{ab}	3.58^{h}	3.02^{ghi}
PA23 (PA23-314)[1]	5.22^{ab}	5.81^{ab}	4.64^{abcd}
PA23-314 (PA23)	5.35^{ab}	5.65^{bc}	4.22^{cdef}
PA23 (PA23-314 *gacS*)	5.50^{ab}	5.56^{c}	4.94^{abcd}
PA23-314 *gacS* (PA23)	5.00^{ab}	5.14^{d}	3.61^{efg}
PA23 (PA23-63)	5.67^{ab}	4.12^{f}	3.26^{fghi}
PA23-63 (PA23)	3.00^{ab}	3.65^{g}	2.77^{ghi}
PA23 (PA23-1)	6.15^{a}	5.12^{d}	5.15^{abc}
PA23-1 (PA23)	4.55^{ab}	3.16^{i}	5.43^{a}
PA23 (PA23-63-1)	5.90^{ab}	3.78^{g}	4.04^{def}
PA23-63-1 (PA23)	4.30^{ab}	3.58^{f}	2.38^{i}
LSD	3.7472	0.2039	0.9921

The bacteria were isolated in nutrient agar (NA) supplemented with specific antibiotics for a particular bacterial strain. The values followed by letters in each column denote the significant groupings resulted from the Fisher's least significant difference test. Values followed by the same letters in a column are not significantly different at $P = 0.05$ significant level according to Fisher's least significant difference test.
[1]The strain included in the parentheses was combined with the strain that is not in parenthesis and log CFU values in each column correspond to the bacterial log CFU values shown by the strain that is not in parenthesis.

seeds. In the rest of the treatments, the bacterial numbers on the roots continued to increase up to four weeks of incubation and showed a drastic reduction in numbers by week six.

Total bacterial count

There was no significant difference ($P = 0.05$) among total bacterial counts in the 2nd and 4th week after sowing (data not shown) and the total bacterial count for each treatment reduced with time.

Introduced bacteria as a percentage of total rhizosphere bacteria

Strain PA23 applied alone or in combination with the other mutants showed comparatively higher percentages than the other strains for all three sampling days. The second highest counts were with strain PA23-314 (Table 3). PA23-314 applied with PA23 maintained relatively higher percentages (10–25%) throughout the sampling period. The contribution of the other introduced strains towards the rhizosphere community was very low; PA23-63-1 combined with PA23 showed the lowest contribution at 0.2%. By week six, most of the strains that produced at least

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Table 3. Introduced *Pseudomonas chlororaphis* PA23 and its various mutants alone or in combinations, re-isolated from 1 g of seminal roots of sunflower, as a percentage of the total bacteria isolated from 1 g of seminal roots treated with each treatment, over a period of 6 weeks after sowing.

Strain	2 weeks	4 weeks	6 weeks
PA23	67.50[a]	11.48[a]	5.54[bc]
PA23-314	44.30[abc]	6.22[abc]	1.58[bc]
PA23-314 *gacS*	9.75[cd]	1.74[cd]	0.26[c]
PA23-63	0.25[d]	0.48[d]	0.30[c]
PA23-1	2.60[d]	0.04[d]	0.53[c]
PA23-63-1	4.55[d]	0.12[d]	0.26[c]
PA23 (PA23-314)[1]	32.20[abcd]	7.64[ab]	11.86[bc]
PA23-314 (PA23)	18.30[bcd]	5.14b[cd]	4.04[bc]
PA23 (PA23-314 *gacS)*	47.45[ab]	4.97[bcd]	22.23[bc]
PA23-314 *gacS* (PA23)	15.15[bcd]	2.10[cd]	0.68[c]
PA23 (PA23-63)	20.80[bcd]	0.04[d]	4.60[bc]
PA23-63 (PA23)	3.85[d]	0.01[d]	5.17[bc]
PA23 (PA23-1)	8.10[d]	3.98[bcd]	60.07[a]
PA23-1 (PA23)	1.05[d]	0.06[d]	10.07[bc]
PA23 (PA23-63-1)	18.75[bcd]	0.12[d]	32.04[ab]
PA23-63-1 (PA23)	0.20[d]	0.20[d]	0.66[c]
LSD	35.754	0.2039	0.9921

Appropriate dilutions were plated onto nutrient agar (NA) plates supplemented with particular antibiotics assigned for each strain to get the introduced bacterial density and onto NA plates without antibiotics to get the total bacterial count. The values followed by letters in each column denote the significant groupings resulted from the Fisher's least significant difference test. Values followed by the same letters in a column are not significantly different at $P = 0.05$ significant level according to Fisher's least significant difference test.

[1]The strain included in the parentheses was combined with the strain that is not in parenthesis and log CFU values in each column correspond to the bacterial log CFU values shown by the strain that is not in parenthesis.

one antibiotic (Table 3) had started to increase their numbers noticeably except strains PA23-314 *gacS*, PA23-63, and PA23-314 *gacS* applied with PA23.

Sclerotinia wilt disease assessment

The results of the three separate experiments were pooled and analyzed together. Percentage seedling emergence after treatment with bacterial strain PA23 was significantly higher than all other bacterial treatments including the positive control except that of PA23 + PA23-1 (Figure 4). The Fungicide (Ronilan) treatment showed the highest percentage emergence and was significantly different from all other treatments except the negative control. All other treatments exhibited a lower percentage seedling emergence than the positive control (Figure 4). The lowest seedling emergence was observed in the treatment PA23-314.

Discussion

This study attempted to understand genetic regulation of organic volatile antibiotic production in the biocontrol strain PA23. Moreover, we were interested in

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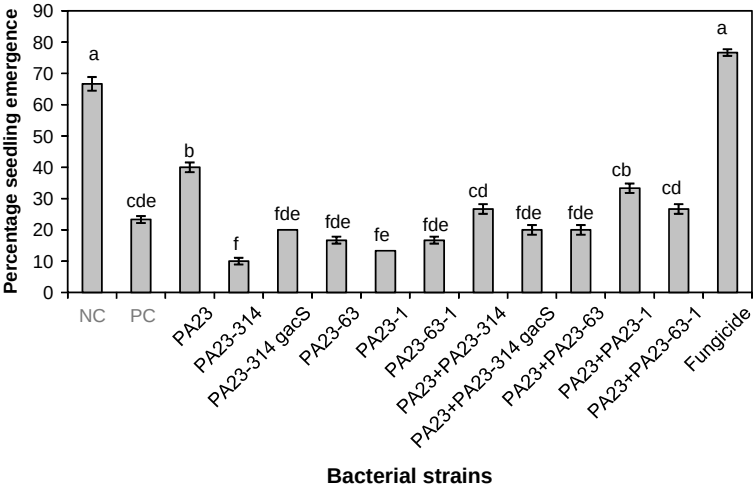

Figure 4. Percentage seedling emergence shown by sunflower seeds treated with different bacteria or bacterial combinations (wild-type + mutant) 2 weeks after sowing. Seeds were treated with 10^8 CFU/ml bacterial suspensions prepared in 1% methycellulose in 0.1 M phosphate buffer (pH 7.0). Bars followed by the same letters are not significantly different, $P = 0.05$, using the Fisher's least significant difference. LSD: 0.1194. NC, negative control; PC, positive control.

elucidating how antibiotic production impacts the ability of PA23 to colonize roots and control *Sclerotinia sclerotiorum* disease of sunflower.

All of the mutants inhibited mycelial growth and sclerotia and ascospore germination as well as or better than the wild-type, with the exception of the two regulatory mutants PA23-314 (*gacS*$^-$) and PA23-443 (*ptrA*$^-$) (Figure 1). However, GC-MS analysis revealed the 3 volatiles nonanal, benzothiazole, and 2-ethyl–1-hexanol were produced by all of the strains, including PA23-314 (*gacS*$^-$) and PA23-443 (*ptrA*$^-$). At present, it is unclear why less fungal inhibition was observed for these two mutants. We speculate that there may be differences in the amounts of the three volatiles produced and since the GC-MS analysis was non quantitative, this difference was not apparent. Alternatively, other volatile antifungal compounds are being produced that can not be detected by the methods employed herein. HCN, for example, is a potent volatile antifungal compound that is produced by PA23. It has been shown that HCN production is inhibited in both bacterial strains PA23-314 and PA23-443, while HCN levels remain unchanged in the other strains (Poritsanos 2005; Selin et al. 2010). Therefore, the elevated inhibition of *S. sclerotiorum* shown by other strains might be the result of HCN accumulation in the headspace of the Petri plate. This is further supported by the fact that for any strains or combination of strains in which the above genes have been restored by complementation, wild-type levels of inhibition were observed. Castric (1983), Laville et al. (1998), and Blumer and Hass (2000) found that the HCN production in *P. aeruginosa* and *P. fluorescens* cultures was optimally induced under oxygen limiting conditions. The sealed Petri plate in this study might have provided the ideal conditions by allowing growth of the bacteria to reduce oxygen levels in the headspace enhancing HCN production. However, HCN could not be detected by GC-MS analysis due to

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its low molecular weight. To our knowledge this is the first time that a study has attempted to examine bacterial strains with mutations for production of organic volatile antibiotics produced by antagonistic bacteria.

With respect to colonization, wild-type strain PA23 showed significantly higher bacterial density over the sampling period (Table 2) compared with the mutants. PA23 was originally isolated from root tips, therefore, its adaptation to root tips may account for its success in colonizing sunflower roots. Gradual reduction of introduced and total bacterial numbers from week 2 to week 6 may be due to insufficient nutrient availability in the soil, since no fertilizer was added to the pots after the initial fertilizer application. In addition, by week 6, sunflower plants had started to form floral buds. This particular time period is critical for the plant reproduction. Waisel, Eshel, and Afkafi (1991) found that the stage of plant development is one of the factors affecting root exudate composition. Therefore changes in the composition of the root exudate together with soil nutrient availability might have contributed to the variation in bacterial numbers on roots observed over the sampling period. This idea is also supported by Botelho and Mendonça-Hagler (2006) and Bloemberg and Lugtenberg (2001) where they stated that the root colonization potential is related to the nutritional balance for the bacteria and the function of genes related to rhizosphere colonization play an important role in this regard. Interestingly, some antibiotic-producing strains showed a sudden increase in their percentages by week 6 (Table 3). It seems that the antibiotic-producing strains started to increase their numbers, possibly prevailing through their production of antibiotics. Mazzola et al. (1992) stated that when resources are plentiful, the production of phenazine would be of little benefit to the producer but the production of phenazine increases when the microbial population is high and resources become limited. This explanation may account for the observations made in the current study. Throughout the sampling period most of the strains that could produce antibiotics, except PA23-63, maintained higher bacterial numbers and percentages (higher than 5%; Table 3) than non-producing strains. PA23-63-1, which produced neither phenazine nor pyrrolnitrin showed the lowest root colonization levels (Table 3). This suggests a trend where the number of antibiotics produced is proportional to the survival of bacteria on roots. Antibiotic deficiency seemed to reduce the ecological fitness of PA23 to various extents independent of the type of mutation introduced. However, there was no distinct pattern in the variation between phenazine-deficient and pyrrolnitrin-deficient mutants observed in the current study. Therefore, the relative importance of phenazine and pyrrolnitrin on the root colonization ability as well as the biocontrol ability of PA23 is unclear. This may be due to the fact that antibiotic production by a BCA can be affected by several biotic and abiotic factors such as oxygen, temperature, specific carbon and nitrogen sources (Howie and Suslow 1991; Ownley, Weller and Thomashow 1992; Shanahan, O'Sullivan, Simpson, Glennon and O'Gara 1992; Slininger and Jackson 1992; Duffy and Défago 1997), plant host (Maurhofer, Keel, Adkins, Walter and Redeout 1992; Georgakopoulos, Hendson, Panopoulos and Schroth 1994), pathogen (Duffy and Défago 1997) and indigenous microflora (Wood and Pierson 1994). Furthermore, the competitive colonization shown by PA23-314 (Table 2) could be attributed to the elevated amounts of siderophores reported to be produced in this strain (Poritsanos et al. 2006).

Combinations of antibiotic producing and/or deficient strains did not show a significant synergistic effect on root colonization and/or Sclerotinia wilt suppression. However, a general trend in reduced root colonization or biocontrol ability of PA23 was observed for some strain combinations (Table 2 and Figure 4). This suggests that the combination of a single antibiotic-producing mutant and PA23 may exert additional competitive pressures, which may reduce the effectiveness of PA23.

In summary, the type of mutations that were examined in the current study did not impact production of the three organic volatile antibiotics produced by PA23. However, they could affect the amount of the antibiotic produced, which was not an objective of this study but an area that should be investigated further. The mutations in PA23, regardless of the type, reduced the efficacy of its root colonization ability and combination of certain mutants with PA23 reduced its effectiveness. Furthermore, the capacity to produce antibiotics appeared to be most advantageous to PA23 when the nutrients became limited in the rhizosphere. Since antibiotic production, root colonization and biocontrol ability are strongly interconnected and influenced by environmental factors, it becomes difficult to determine the importance of one factor over another in the control of soil-borne plant pathogens. Regardless, studies such as these, focused on understanding the regulation of biocontrol factors and their contribution to environmental fitness and pathogen control are essential for the development of rational strategies for antagonist application, as well as augmenting desirable characteristics through genetic engineering.

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