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Pseudomonas chlororaphis Strain Sm3, Bacterial Antagonist of *Pratylenchus penetrans*

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Abstract: The interaction of *Pseudomonas chlororaphis* strain Sm3 and the root-lesion nematode *Pratylenchus penetrans* was investigated in three separate greenhouse experiments with soils from southern British Columbia, Canada. The bacteria were applied to the roots of strawberry plants and planted in unpasteurized field soils, with natural or supplemented infestation of *P. penetrans*. Nematode suppression in roots was evident after 6 or 10 weeks in all experiments. Root or shoot growth were increased after 10 weeks in two experiments. Population dynamics of *P. chlororaphis* Sm3 in the rhizosphere was followed using an antibiotic-resistant mutant of *P. chlororaphis* Sm3. There was no apparent correlation between bacterial density in the rhizosphere and *P. penetrans* suppression in strawberry roots and rhizosphere soil, although the soil with the highest nematode reduction also had the largest *P. chlororaphis* Sm3 population in the rhizosphere.

Key words: bacteria, biological control, growth promotion, nematode, *Pratylenchus penetrans, Pseudomonas chlororaphis,* rhizosphere bacteria, root-lesion nematode, strawberry.

The root lesion nematode, *Pratylenchus penetrans,* is a common endoparasitic pest of a large number of crops in temperate climates. *Pratylenchus penetrans* also promotes various disease complexes with fungal pathogens in many crops, including strawberry (*Fragaria x ananassa* Duchesne) (Chen and Rich, 1962; Kurppa and Vrain, 1989). Bacteria with a potential to suppress plant-parasitic nematodes have been isolated from potatoes, tomatoes, peach trees, and others (Cronin et al., 1997; Racke and Sikora, 1992; Spiegel et al., 1991; Westcott and Kluepfel, 1993). However, except for the nematode-parasitic *Pasteuria penetrans* (Stirling, 1991), bacterial antagonists of *P. penetrans* have been described only from in vitro screens (Walker et al., 1966) and have not been tested in field soil (Hackenberg et al., 1997).

Several strains of *Pseudomonas chlororaphis* protect roots from soilborne pathogenic fungi (Cartwright and Benson, 1995; Duffy et al., 1996; Johnsson et al., 1998; Knudsen et al., 1997; Kropp et al., 1997; Landa et al., 1997; Chin-A-Woeng et al., 1998). *Pseudomonas chlororaphis* Sm3 was found to promote the growth of conifer seedlings (Shishido et al., 1996). With apple seedlings, *P. chlororaphis* Sm3 reduced *P. penetrans* numbers per root system by 61% in a preliminary screen, and nematode numbers per gram of root by 41% in a repeated screen (unpubl.). The objective of this study was to assess the antagonism of *P. chlororaphis* Sm3 toward *P. penetrans* parasitizing strawberry plants growing in the greenhouse in non-pasteurized field soils from British Columbia.

Materials and Methods

Effects of P. chlororaphis and nematode inoculum levels on plant growth (experiment 1): The effect of *P. chlororaphis* Sm3 on growth of strawberry plants was tested in a loamy sand taken from a field of the Pacific Agri-Food Research Centre in Summerland, British Columbia (Summerland a). This soil was not pasteurized prior to the experiment and was naturally infested with 1,500 *P. penetrans*/liter (Table 1). Half of the pots received an additional 1,000 *P. penetrans*/liter soil. The *P. penetrans* inoculum, originally from a raspberry field near Abbotsford, British Columbia, was multiplied in monoxenic corn root cultures on Gamborg's B5 medium (Huettel and Rebois, 1985). Nematodes were extracted from 4-month-old corn roots in Baermann pans, and collected every 24 hours for 3 days. Inoculum of *P. penetrans*

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TABLE 1. Composition of soils and substrates used in experiments 1, 2, and 3, and initial *Pratylenchus penetrans* densities.

Soil	Experiment	pH	Conductivity (dS/m)	Organic matter (%)	Sand (%)	Silt (%)	Clay (%)	*P. penetrans* per liter
Summerland a	1	7.7	—	1.2	—	—	—	1,500
Delta soil mix	2	5.8	2.24	11.7	51.5	34.5	14.0	0.0
Summerland b	3	7.6	0.36	0.5	90.5	7.5	2.0	144
Chilliwack	3	5.5	0.44	3.8	11.0	72.5	16.5	214
Langley b	3	5.6	0.40	10.5	53.5	38.5	8.0	803
Langley p	3	5.3	0.44	9.2	51.0	40.0	9.0	187
Abbotsford	3	5.4	0.16	1.8	82.0	13.0	5.0	37
Aldergrove	3	5.3	1.20	5.8	54.0	37.5	8.5	30

— Data not obtained.

was adjusted to 1,000/10 ml water. *Pseudomonas chlororaphis* Sm3 was routinely grown on King's medium B (KB) (King et al., 1954), pH 7.2–7.4 at 28 °C, and stored as needed in 50% glycerol at −70 °C. For the preparation of inocula for experiments, the *P. chlororaphis* Sm3 was grown in liquid minimum medium (modified M9, Sambrook et al., 1989) containing 0.5 g $(NH_4)P_3O_5$ with 0.1 g KCl, 0.1 g $MgSO_4$, 0.5 g yeast extract, and 2 g sucrose/liter. The cultures were shaken at 100 rpm at room temperature in darkness for 24 hours and formed ca 10^9 colony-forming units per milliliter (cfu/ml). Plastic pots (15-cm diam.) received 250 ml of *P. penetrans*-infested loamy sand (Summerland a), and 10 ml of nematode inoculum (1,000 *P. penetrans*) or 10 ml of water was pipeted on top of the sand. Strawberry plants (cv. Totem) were grown from runners in a peat-based potting mix in the greenhouse. The soil mix was removed, and one strawberry plant with bare roots was placed on top of the sand in each pot. Ten ml of a 24-hour-old *P. chlororaphis* Sm3 culture (10^9 cfu/ml) in liquid minimum medium was pipeted on the roots, and 10 ml of sterile minimum medium was pipeted on roots of control plants. The roots were covered with 250 ml of the same loamy sand (Summerland a); therefore, the total *P. penetrans* inoculum was either 750/plant or 1,750/plant. The pots were placed in a greenhouse in a two-factor completely randomized design with 12 replicate plants per treatment. Temperature regime was 24 °C during the day and 16 °C at night with a 16-hour photoperiod. The plants were watered daily as needed, and fertilized once with 20:20:20 NPK at 0.02 gN/plant. After 6 weeks, fresh weights of roots and shoots were determined. Nematodes were extracted from a portion of the roots of each plant in a mist chamber for 7 days, and from the soil of each pot in Baermann pans for 7 days. Numbers of *P. penetrans* per gram of roots and per 50 ml of soil were determined, and numbers per root system and pot were calculated. The data were analyzed with a two-factor ANOVA. Nematode numbers were log-transformed before ANOVA, and least significant differences (LSD) were calculated for mean comparisons.

Effects of P. chlororaphis Sm3 on plant growth and nematode density over time (experiment 2): Plastic pots (12.5-cm diam.) were half-filled with an unpasteurized soil mix (soil:peat:perlite, 1:1:1) made from a silt loam field soil (Delta Soil Mix, Table 1) from Delta, British Columbia. The *P. penetrans* inoculum consisted of nematode-infested pieces of raspberry roots (<2 cm long) mixed with the soil in which the raspberry plants had grown. The inoculum was calibrated by extracting roots and soil in Baermann pans for 7 days. An estimated 1,500 nematodes in raspberry root pieces (equivalent to approximately 0.25 g of dried root weight) and soil were placed on top of the soil mix. Rooted strawberry plants were placed on top of the soil mix and nematode inoculum, and each pot was filled to 1 liter. The bacterial inoculum (*P. chlororaphis* Sm3) was pipeted on top of the soil in the same manner as in experiment 1. Pots were placed in a greenhouse at

a temperature regime of 22 °C during the day and 17 °C at night with a 16-hour photoperiod. The plants were watered daily as needed and fertilized twice as described above. The experiment was a completely randomized design with 10 replicate pots for each combination of *P. chlororaphis* Sm3 treatment and harvest time. Half of the plants were harvested after 6 weeks and the remainder after 12 weeks. Root weights and shoot weights were measured, and nematodes were extracted from roots and soil and counted, as in experiment 1. Survival and abundance of *P. chlororaphis* Sm3 in the strawberry rhizosphere was assessed using a spontaneous mutant of the bacterium showing selective resistance to rifampicin and nalidixic acid. This mutant strain was grown on KB medium with the addition of 100 µg nalidixic acid and 100 µg rifampicin/liter. Three extra strawberry plants were harvested 3, 6, 9, and 12 weeks after bacterial inoculation of roots and soil. Roots were separated from the plants, and a 1-g aliquot was placed in 100 ml of 0.1 M $MgSO_4$ and shaken (150 rpm) for 20 minutes. Colonies of *P. chlororaphis* Sm3 were counted after dilution plating on KB medium and incubation at 25 °C for 24 to 48 hours.

Effects of P. chlororaphis Sm3 on plant growth and nematode population density in unpasteurized field soil (experiment 3): Initial *P. penetrans* densities were determined for six soils from British Columbia (Table 1). Paper pots (15-cm diam.) were half-filled with these soils and infested with 900 *P. penetrans* from monoxenic corn root culture as described in experiment 1. *Pseudomonas chlororaphis* Sm3 was grown in liquid KB medium in petri dishes (100 rpm; 28 °C). After 24 hours the bacterial cells were separated from the growth medium by centrifugation (10 minutes at 4,000 rpm), and resuspended in 50 ml of phosphate buffered saline (PBS: 1.18 g Na_2HPO_4, 0.22 g $NaH_2PO_4 \cdot H_2O$, 8.5 g NaCl/liter distilled water, pH 7.2). Strawberry plants (cv. Totem) from tissue culture were rooted and placed in pots as in experiment 1. Five ml of *P. chlororaphis* Sm3 suspension (1.5×10^{11} cfu in PBS) were distributed onto each strawberry root system with a pipet, and soils were added to 1 liter in each pot. Control plants were treated with 5 ml of sterile PBS. Ten pots/treatment were placed in a greenhouse in a randomized complete block design, with pots blocked according to initial plant size. Plants were fertilized every 2 weeks with 9 mg N/4.5 mg P/13.5 mg K per pot and watered daily. Temperature was 24 °C during the day and 16 °C at night with a 16-hour photoperiod. After 10 weeks, root and shoot fresh weights were recorded, and nematodes were extracted and counted as described above. Statistical analyses were as in the first experiment. *Pseudomonas chlororaphis* Sm3 population density and *P. penetrans* per root system and per gram of roots were calculated.

Results and Discussion

Nematode numbers per gram of roots treated with *P. chlororaphis* Sm3 were reduced after 6 weeks by 47% and 43% in experiments 1 and 2, respectively (Tables 2, 3). Neither the main-factor effect of *P. chlororaphis* Sm3 inoculation nor the interaction between *P. chlororaphis* Sm3 inoculation and initial nematode population density was significant for the numbers of *P. penetrans* per root system or per pot (Table 2). In experiment 1, the interaction between *P. chlororaphis* Sm3 inoculation and *P. penetrans* inocula levels was significant ($P = 0.02$) for *P. penetrans* per gram of root (Table 2). After 6 weeks in experiment 2, nematode numbers per gram of roots, per root system, and per pot were all reduced by *P. chlororaphis* Sm3 inoculation compared to the control (Table 3), but the effect of *P. chlororaphis* Sm3 was not significant after 12 weeks (Table 3). Root and shoot growth of plants inoculated with 1,750 nematodes and treated with *P. chlororaphis* Sm3 were increased after 6 weeks by 54% and 36%, respectively ($P = 0.05$), in experiment 1, but there was no significant root or shoot growth increase due to *P. chlororaphis* Sm3 when plants were inoculated with only 750 nematodes (Table 2). The interaction between *P. chlororaphis* Sm3 and *P. penetrans* inocula levels was sig-

TABLE 2. Effects of *Pseudomonas chlororaphis* Sm3 on population densities of *Pratylenchus penetrans* and growth of strawberry plants in soil naturally infested with *P. penetrans* (750 *P. penetrans*/pot) and in soil infested with an additional 1,000 *P. penetrans.*

Nematode inoculum per pot	Bacteria added	*Pratylenchus penetrans* Per gram root	Per root system	Per pot	Plant dry weight (g) Roots	Shoots
750	−	141 b	412 b	539 b	3.5 ab	2.7 ab
	+	258 b	644 b	847 b	2.7 b	2.6 ab
1,750	−	1,138 a	2,328 a	2,451 a	2.6 b	2.2 b
	+	598 b	2,169 a	2,362 a	4.0 a	3.0 a
P-value (Sm3)		0.85	0.81	0.37	0.40	0.09
P-value (*P. penetrans*)		<0.001	<0.001	<0.001	0.65	0.84
P-value (Sm3 × *P. penetrans*)		0.02	0.33	0.15	0.01	0.04

Values within a column followed by the same letter are not significantly different according to Fisher's protected LSD ($P = 0.06$).

nificant for both root ($P = 0.01$) and shoot ($P = 0.04$) dry weights (Table 2). After 12 weeks, root and shoot growth of plants treated with *P. chlororaphis* Sm3 in experiment 2 were increased by 32% and 18% respectively, but the increase was not significant (Table 3). Populations of the rifampicin and nalidixic acid-resistant strain of *P. chlororaphis* Sm3 decreased between 3 and 12 weeks in experiment 2 (Fig. 1).

Nematode population densities at harvest varied significantly among the six soils of experiment 3, but, on average, inoculation with *P. chlororaphis* Sm3 reduced *P. penetrans* per gram of root, per root system, and per pot by 24%, 30%, and 29%, respectively (Table 4). Main-factor effects of *P. chlororaphis* Sm3 inoculation and soil type were both significant ($P \leq 0.01$) for *P. penetrans* per gram of root, per root system, and per pot (Table 4). The interaction between *P. chlororaphis* Sm3 inoculation and soil type was not significant for any nematode dependent variable (Table 4). Plant growth was not correlated with nematode population densities for *P. chlororaphis* Sm3-inoculated, uninoculated, or combined data sets. *Pseudomonas chlororaphis* Sm3 populations ranged between 1.7×10^7 and 2.6×10^8 cfu in the rhizosphere of strawberry plants growing in the six British Columbia soils 10 weeks after bacterial inoculation. When averaged over the six soils, inoculation with *P. chlororaphis* Sm3 increased shoot weight (Table 4; $P < 0.09$). Soil type also had a significant effect on both root and shoot dry weights (Table 4; $P < 0.001$), but the interaction between *P. chlororaphis* Sm3 and soil type was not significant for root or shoot dry weights.

This is the first report of *P. chlororaphis* antagonism toward a plant-parasitic nematode. Other *Pseudomonas* spp. have been reported to be antagonistic toward *Meloidogyne incognita* (Becker et al., 1988) and *Heterodera schachtii* (Oostendorp and Sikora, 1989). *Pseudomonas chlororaphis* Sm3 was originally

TABLE 3. Effects of *Pseudomonas chlororaphis* Sm3, 6 and 12 weeks after inoculation, on population densities of *Pratylenchus penetrans* and growth of strawberry plants in soil infested with 1,500 *P. penetrans.*

Time after inoculation	Bacteria added	*Pratylenchus penetrans* Per gram of root	Per root system	Per pot	Plant dry weight (g) Roots	Shoots
6 weeks						
	−	507	3,454	4,665	7.25	27.79
	+	290*	2,217*	3,389*	7.84	27.42
12 weeks						
	−	563	6,149	8,041	10.71	35.87
	+	423	5,740	7,582	14.15	42.26

* Values labelled with an asterisk are significantly different from non-inoculated controls (T-test, $P = 0.05$).

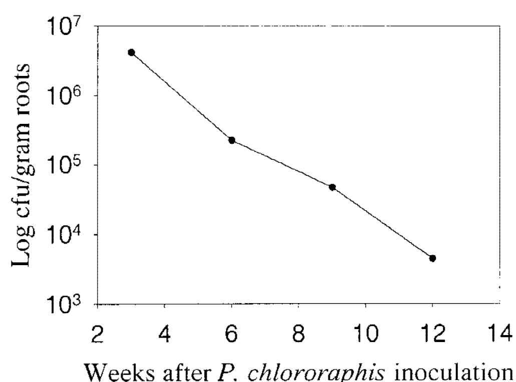

Fig. 1. Density of *Pseudomonas chlororaphis* Sm3 on strawberry roots (cfu/gram of roots) in time (experiment 2).

isolated from the rhizosphere of *Pseudotsuga menziesii* (Douglas-fir) and has been described as a general growth promoter of conifer seedlings (Chanway et al., 1997; Shishido et al., 1996). In this study, *P. chlororaphis* Sm3 reduced nematode numbers and increased the growth of strawberry plants, suggesting that the positive effects on conifer seedling growth could be the result, in part, of suppression of plant-parasitic nematodes or other soilborne pathogens.

In preliminary tests in our laboratory, *P. chlororaphis* Sm3 was antagonistic toward *Botrytis* sp., *Pythium ultimum, Fusarium* sp., *Pestalotia* sp., *Physalospora vaccinii,* and *Allantophomopsis lycopodina* (data not shown) but had no effect on *P. penetrans* activity or mortality in vitro. Our experiments did not demonstrate the mechanism by which *P. chlororaphis* Sm3 reduced population growth of *P. penetrans* in strawberry roots. Possible mechanisms of antagonism of rhizosphere bacteria toward plant-parasitic nematodes include the production of toxic metabolites or antibiotics in the rhizosphere, and systemic acquired resistance (Hasky-Gunther et al., 1998; Martinez-Ochoa et al., 1995). Successful biological control of fungal pathogens by fluorescent pseudomonads is often associated with the production of toxic metabolites, antibiotics, or siderophores (Weller and Tomashow, 1993), although systemic acquired resistance has also been demonstrated (Kloepper et al., 1993). Regardless of the mechanisms of antagonism, colonization of the rhizosphere for an extended period is generally considered to be necessary for effective biological control (Defago and Keel, 1993; Weller, 1988). This study did not demonstrate whether *P. chlororaphis* Sm3 could successfully colonize the rhizosphere of strawberry plants and reduce *P. penetrans* for a season. Population densities of *P. chlororaphis* Sm3 decreased mono-

Table 4. Effects of *Pseudomonas chlororaphis* Sm3 and soil type on population densities of *Pratylenchus penetrans* and growth of strawberry plants 10 weeks after planting and inoculation with 1.5×10^{11} cells of the bacterium in phosphate-buffered saline.

Bacteria added	*Pratylenchus penetrans*				Plant dry weight (g)	
	Pi[1]	Per gram root	Per root system	Per pot[2]	Roots	Shoots
+		70	488	751	1.10	3.50
–		92	701	1,059	1.33	3.35
P-value (main-factor effect)		0.004	0.003	0.001	<0.001	0.09
Soil						
Langley b	1,703	158 a[3]	1,127 a	1,768 a	1.06 d	2.45 c
Langley p	1,087	141 a	874 a	1,529 ab	0.88 de	2.55 c
Abbotsford	1,271	74 b	747 a	1,061 b	1.46 b	3.20 b
Summerland	1,044	42 c	404 b	497 c	1.27 c	2.66 c
Chilliwack	1,114	59 bc	267 bc	391 c	0.72 e	2.41 c
Aldergrove	930	14 d	166 c	230 d	1.98 a	7.19 a
P-value (main-factor effect)		<0.001	<0.001	<0.001	<0.001	<0.001
P-value (Sm3 × soil interaction)		0.60	0.36	0.75	0.21	0.42

[1] All pots were infested with 900 *Pratylenchus penetrans* in water. Listed Pi values are the sum of estimates of indigenous population densities, determined via Baermann pan extraction, and the additional 900 nematodes.

[2] Sum of nematodes extracted from the entire root system and in soil.

[3] Values within a column followed by the same letter are not significantly different according to Fisher's protected LSD ($P = 0.05$).

tonically during the course of this study, and there was no apparent stabilization of bacterial population densities at an equilibrium level. In addition, there was no correlation, across different soils, between rhizosphere population densities of *P. chlororaphis* Sm3 and suppression of *P. penetrans*. The direct inoculation of roots increased 10-fold the establishment of *P. chlororaphis* Sm3 in the rhizosphere in experiment 3 as compared to pipeting the bacterial inoculum on top of the soil in the second experiment.

The biological control of soilborne pathogens by rhizosphere bacteria is notoriously sensitive to variation in experimental conditions (Deacon, 1991; Weller, 1988). Previous research indicated that the conifer growth promotion effect of *P. chlororaphis* Sm3 varied among sites and was associated with colonization by certain ecto-mycorrhizal fungi (Shishido et al., 1996). We tested the effects of *P. chlororaphis* Sm3 on *P. penetrans* in a variety of soils to evaluate the sensitivity of *P. chlororaphis* Sm3 antagonism to edaphic conditions. The suppression of *P. penetrans* in all tested soils suggests that antagonism of *P. chlororaphis* Sm3 toward *P. penetrans* may be a relatively robust phenomenon and that *P. chlororaphis* Sm3 may be effective under field conditions.

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