In-vitro interrelationships between rhizosphere bacteria and *Heterodera schachtii*

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SUMMARY

The influence of rhizobacteria on hatching, migration, and penetration of *Heterodera schachtii* was evaluated under laboratory conditions. Application of bacteria to the root surface of sugar beet seedlings did not alter the migration of *H. schachtii* second-stage juveniles towards the root. The hatch stimulating activity of root exudates of sugar beets was reduced (P = 0.05) after treatment of exudates with seven of eight isolates tested. Nematode penetration into the root was reduced (P = 0.05) by six of the eight isolates. Bacterial metabolites were not toxic to *Panagrellus redivivus*. The results indicate marked differences in the mode-of-action of different bacterial isolates.

RÉSUMÉ

Interactions in vitro *entre bactéries de la rhizosphère et* Heterodera schachtii

L'influence des rhizobactéries sur l'éclosion, le déplacement et la pénétration de *Heterodera schachtii* a été évaluée dans les conditions du laboratoire. L'application de bactéries à la surface des racines de plantules de betteraves à sucre n'altère pas le déplacement des juvéniles de deuxième stade de *H. schachtii* vers la racine. La stimulation d'éclosion causée par les exsudats radiculaires de betterave à sucre est réduite (P = 0,05) après traitement de ces exsudats par sept des huit isolats testés. La pénétration du nématode est diminuée (P = 0,05) par six des huit isolats. Les métabolites bactériens ne sont pas toxiques envers *Panagrellus redivivus*. Les résultats obtenus révèlent des différences marquées dans le mode d'action des différents isolats bactériens.

Biological control of plant-parasitic nematodes is often accomplished by the introduction of predators or parasites or by their activation with organic amendments. Some of these biological control systems were studied in detail in greenhouse or field studies (Stirling, 1988). Biological control can also be accomplished by taking advantage of various forms of antagonism that indirectly inhibit nematode development and do not cause direct nematode mortality. These antagonistic interrelationships exist, for example, between nematodes and certain endomycorrhizal fungi (Sikora, 1978, 1981; Hussey & Roncadori, 1982). Saprophytic bacteria applied as a seed or tuber treatment were shown to inhibit early root penetration of *Heterodera schachtii* and *Globodera pallida* in sugar beet and potato, respectively, under greenhouse and field conditions (Racke & Sikora, 1985; Oostendorp & Sikora, 1989). *Meloidogyne incognita* galling on tomato, cucumber, and clover was suppressed following application of bacterial soil drenches or root treatments in greenhouse studies (Zavaleta-Meija & Van Gundy, 1982; Becker *et al.*, 1988). Similar results were obtained in greenhouse tests on cotton, tomato, peanut, and sugar beet treated with *Bacillus subtilis* to control *M. incognita, M. arenaria* and *Rotylenchulus reniformis* (Sikora, 1988).

In this study, we examined possible mechanisms of the antagonistic interrelationship between *H. schachtii* and eight isolates of rhizobacteria from sugar best.

Material and methods

BACTERIA

The bacterial isolates used in this study are designated

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A-3, A-57, A-59, T-58, P-510, P-523, P-76, P-741, and SR-3. Isolates A-59, T-58, and P-523 have been identified as *Pseudomonas fluorescens* (Trevisan) Migula. SR-3 is a Streptomycin-resistant mutant of A-59. All isolates are Gram-negative rods.

All isolates were isolated from the rhizosphere of sugar beet. They were selected for their ability to reduce *H. schachtii* root penetration in greenhouse experiments when used as a seed treatment (Oostendorp & Sikora, 1989).

Bacteria were grown for 18-24 hours at 25° C on the media and at the pH values given in Table 1. The bacterial cells were scrapped off the surface of the agar plates with a sterile spatula and suspended in sterile $MgSO_4$ (0.1 M/l). The controls were treated with 0.1 M/l $MgSO_4$ only.

Table 1

Growth medium and pH of media used for inoculum production of bacterial isolates.

Isolate	*Medium*	*pH*	*Isolate*	*Medium*	*pH*
A-3	St-I	7.2	A-57	St-I	5.6
A-59	St-I	5.6	T-58	TS	5.6
P-510	KB	5.6	P-523	KB	5.6
P-76	KB	7.2	P-741	KB	7.2
SR-3	St-I	5.6 + 100 µg/ml Streptomycin			

St-I = Standard I Agar (Merck)
KB = King's Medium B (King, Ward & Raney, 1954)
TS = Tryptic Soy Agar (Difco)

RHIZOSPHERE COLONIZATION

Sugar beet seeds, *Beta vulgaris* cv. Kawevera were inoculated by submerging the seeds in a bacterial suspension containing isolate SR-3 or in $MgSO_4$ in case of controls. The use of the Streptomycin resistant mutant allowed an estimate of the number of bacteria colonizing the rhizosphere from the seed. Inoculum density was approximately 6.5 × 10^7 colony forming units (cfu)/seed. The average number of cfu/seed was determined by examination of ten seeds treated with bacteria by washing the seeds and dilution plate counting. The rhizobacteria treated seeds and $MgSO_4$ treated control seeds were planted in an unsterilized sand soil mixture (1 : 1 v/v). Five treated plants and five untreated plants were harvested 1, 7, 15, and 22 days after planting. The total number of cfu and the number of Streptomycin resistant cfu found on 1-cm sections of the hypocotyl immediately above the seed, of the main root, of the primary root tip, and of a side root of each plant were counted. On the first day, remaining bacteria on the seed were counted. The bacteria on the plant segments were washed off by vigorous shaking in sterile $MgSO_4$. The resulting suspensions were diluted and aliquots were streaked onto Standard I - Agar with and without Streptomycin. The number of colonies was counted after 48 hours of incubation.

HATCHING TEST

Root exudates were collected by placing 100 six-week-old sugar beet seedlings into a beaker with 1 l of deionized water. The plants were removed after 24 hours and the solution containing the root exudates was sterilized by filtration through a 0.45 µm Millipore filter. Five times nine milliliters of the solution were each mixed with 1 ml of a suspension containing approximately 10^7 cells of each bacterial isolate tested. After 24 hours the bacteria were removed by filtration and the hatch promoting activity of the treated exudates was measured by mixing equal volumes of exudate and a *H. schachtii* egg suspension. Untreated root exudates and water, both amended with the same amount of $MgSO_4$ used to add the bacteria, served as controls. After four days, the number of hatched juveniles and remaining eggs were counted and the hatch rate was calculated according to hatch rate = hatched juveniles × 100/eggs + juveniles.

ATTRACTION TEST

For the attraction tests, the bacteria were inoculated in deionized water instead of $MgSO_4$ as the salt was strongly attractive to the nematode. The bacterial isolates tested showed no loss of viability in water in a preliminary test. The roots of five sugar beet seedlings, grown in sterilized sand in a greenhouse, were dipped into suspensions of each isolate containing 10^8 cells/ml. Approximately 0.1 ml of the suspensions adhered to each root system, thus the bacterial inoculum of each plant was 10^7 cells.

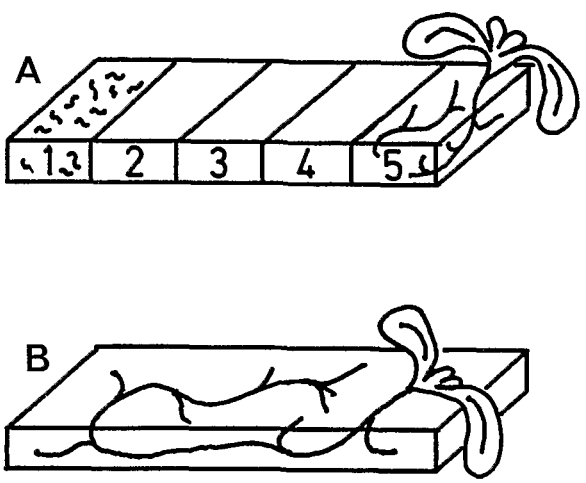

Fig. 1. Sand block chamber for (A) attraction and (B) penetration observations.

The seedlings were planted at one end of 5-cm long × 2-cm wide × 0.5-cm deep block of fine sand (Kerstan & Röpke, 1977). One thousand *H. schachtii* juveniles were then pipetted onto the opposite end of the sand strip (Fig. 1 A). The sand blocks were then moistened with 2 ml of distilled water and incubated in a closed Petri dish at 20° C with 16 h photoperiod for 4 days. The sand blocks were separated into five 1-cm-long sections with a scalpel. The number of juveniles per section was determined by separating the nematodes from the sand by flotation and decanting.

The average distance covered by one juvenile was calculated from juveniles per section and distance travelled from site of inoculation. The number of juveniles that penetrated the root at the end of the block was counted after staining the roots with acid-fuchsin in lactic acid (Ferris, 1985). Untreated plants and sand blocks without plants were used as controls.

Penetration test

The techniques used were the same as in the attraction test. The plant roots, however, were buried along the entire length of the block (Fig. 1 B). Furthermore, 500 nematodes were spread over the entire surface of the block. Juveniles inside the root and in the sand were counted after two days.

Test for toxin production

For the test on possible production of toxic or inhibitory substances, the bacteriophagous nematode *Panagrellus redivivus* was used. The nematode was extracted and inoculated onto agar plates, on which the test bacteria had been grown for 24 h. The activity of the nematode was observed at regular intervals for 48 h.

Results

Naturally occurring Streptomycin-resistant bacteria were only rarely detected in the soil used for the experiment. Therefore, colonies developing on Streptomycin amended agar that had identical colony morphology as SR-3 were considered to originate from the inoculated strain SR-3.

The density of SR-3 on the root surface decreased steadily in the first fifteen days after sowing. The number was below detectable levels on some parts of the root systems. Higher population densities of SR-3, however, were found on four out of five root systems 22 days after inoculation. SR-3 populations ranged from 1.2×10^3 to 10^5 cfu/mm hypocotyl immediately above the seed. This was equivalent to 50 to 100 % of the total bacterial population counted. Extreme variability was observed in the density of SR-3 on primary and secondary roots. Numbers of SR-3 ranged between 0 and

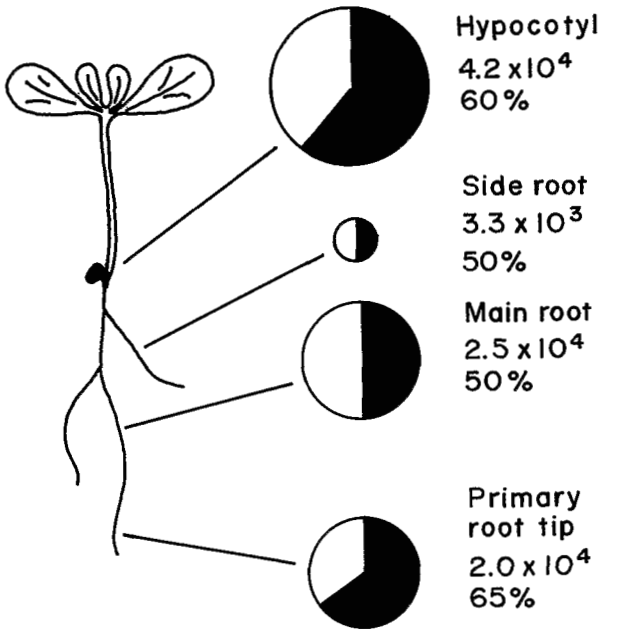

Fig. 2. Density of rhizobacterial colonization of sugar beet roots in cfu/mm and in percent of the total bacterial flora 22 days after seeding.

10^5 cfu/mm of root, the latter representing 100 % of the total bacterial population measured. Examination of the tips of the primary roots revealed bacterial densities that ranged between 2.7×10^3 and 10^5 cfu/mm root tip. The average numbers of bacteria and percentage SR-3 on different parts of the root systems 22 days after sowing are given in Fig. 2.

Hatching

Seven of the eight isolates tested suppressed root exudate hatching activity ($P \leqslant 0.05$) compared to untreated root exudates (Fig. 3). Isolate P-741 reduced hatching 15 % which was equivalent to the hatch in the water control. Isolate P-523 did not reduce the hatch promoting activity of the root exudates.

Attraction

The eight bacterial isolates tested did not influence the migration of *H. schachtii* juveniles to the roots. The juveniles migrated an average 2 cm through the sand blocks towards the roots in the controls and the bacterial treatments. Migration away from the inoculation site was less than 1 cm in the controls without plants (Fig. 4).

Penetration

Six of the isolates tested caused decreases ($P \leqslant 0.05$) in the penetration levels when compared to the un-

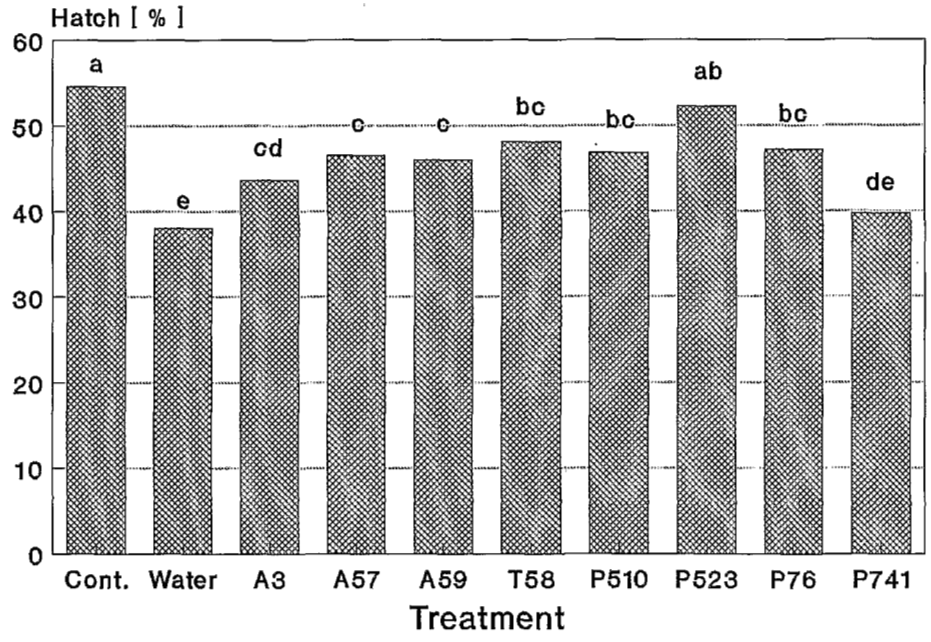

Fig. 3. Influence of rhizosphere bacteria treatment of sugar beet root exudates on *Heterodera schachtii* hatch after 4 days. Columns with different letters are significantly ($P \leq 0.05$) different according to Duncan's Multiple Range Test.

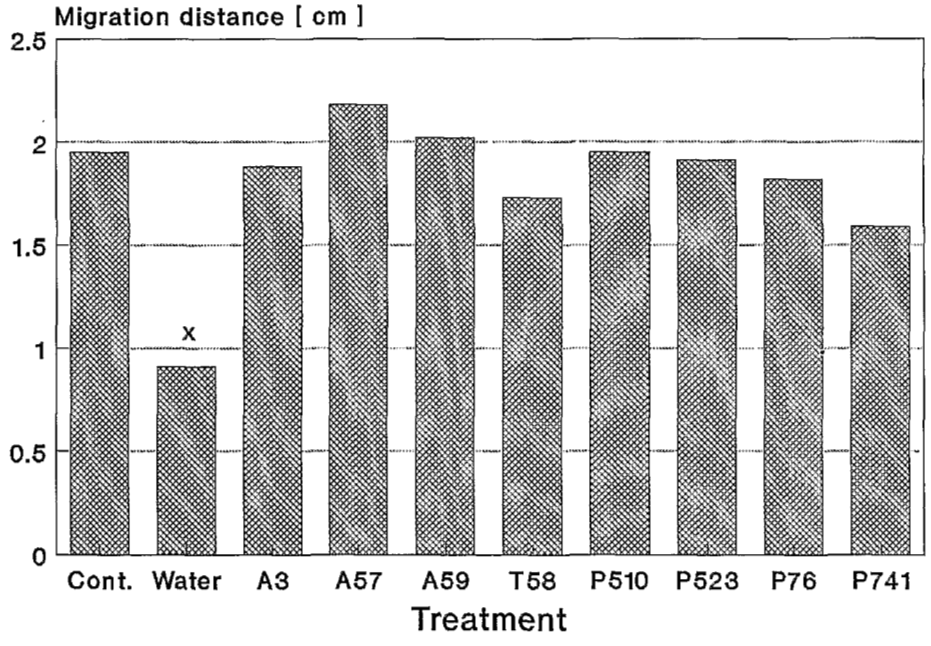

Fig. 4. Influence of treatment of sugar beet roots with rhizobacteria on attraction of *Heterodera schachtii* juveniles to the roots. Significant differences ($P \leq 0.05$) from the control according to Duncan's Multiple Range Test are marked x.

treated controls (Fig. 5). Penetration was reduced ($P \leqslant 0.001$) 55 % and 68 % under the control level when roots were treated with isolates A-57 and P-523, respectively. Isolates P-76 and P-741 did not alter penetration levels.

TOXIN PRODUCTION

None of the bacteria tested produced metabolites inhibitory or toxic to *Panagrellus redivivus*. There were no indications that the bacteria altered the movement or behavior of the nematode.

Discussion

Fluorescent pseudomonads are frequently used in the biological control of soil-borne plant-pathogenic fungi. The production of antibiotics and the competition for iron by the release of siderophores were shown to be active mechanisms of the control (Howell & Stipanovic, 1980; Loper, 1988). The mechanism responsible for the reduction in *H. schachtii* penetration in sugar beet following seed treatment with rhizobacteria have not yet been studied in detail.

The observed decline of the population of isolate SR-3 in the rhizosphere during the first two weeks after sowing indicates that the inoculation technique of the bacteria is not satisfactory. Large differences in the level of colonization were observed between plants. Although the wildtype strain A-59 may be more competitive than the mutant strain SR-3, we believe that an inoculation technique must be developed that extends bacterial survival and aids a more reliable colonization.

The number of antagonistic rhizobacteria detected 22 days after planting agrees with the findings of Suslow and Schroth (1982). From these numbers and from an estimate of the root length observed in this experiment, we concluded that 10^7 cfu per root system is well in the range that occurs in unsterilized soil. Therefore, one plant or its exudates were treated with approximately this number of bacteria in the experiments on the mode-of-action to reduce possible side effects of the presence of untypically high numbers of bacteria.

Root exudate hatch stimulation was reduced ($P \leqslant 0.05$) by incubation with seven of the eight isolates. Reduction to the level considered to be spontaneous hatch occurred with isolate P-741. There was no reduction below the level of spontaneous hatch in water. These results may be interpreted as a bacterial degradation of the components of the exudates that stimulate hatching. Similarly, Utkhede and Rahe (1980) discuss the degradation of *Sclerotium cepivorum* germination stimuli by *Bacillus subtilis*. A complete inhibition of *H. schachtii* hatch by bacterial culture filtrates has also been observed (Bergmann & Van Duuren, 1959).

An interaction between root surface lectins and surface carbohydrates of the nematode may be a prerequi-

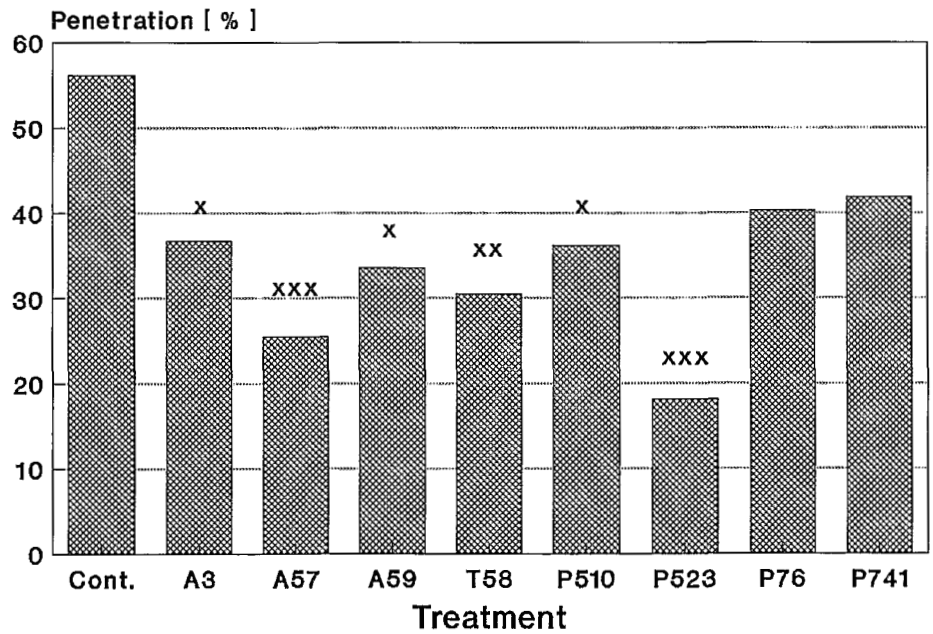

Fig. 5. Influence of treatment of sugar beet roots with different rhizobacterial isolates on penetration of *Heterodera schachtii* juveniles. Significant differences from the control according to Duncan's Multiple Range Test are marked x ($P \leqslant 0.05$), xx ($P \leqslant 0.01$), and xxx ($P \leqslant 0.001$).

site for penetration (Zuckerman & Jansson, 1984). The bacteria tested in this study were all Gram-negative and may have lectin binding structures in the lipopolysaccharide layer of the cell wall membrane (Lotan, Sharon & Mirelman, 1975). Therefore, the mechanism responsible for the reduction in penetration may be related to the ability of the bacteria to envelop or bind to root surface lectins, thereby interacting with normal host recognition.

The production of nematicidal compounds by rhizobacteria has been observed in experiments to control *M. incognita* (Becker *et al.*, 1988). No toxic effects of the bacteria were observed in our bioassay system with the isolates tested.

Rhizobacteria have effectively reduced nematode penetration in greenhouse and field experiments (Oostendorp & Sikora, 1989). The level of control presently achieved cannot be compared to the high levels obtained with nematicides and varied greatly between years, but rhizosphere bacteria may offer plant protection an alternative biological control agent. Future research should be directed to finding more effective strains antagonistic to nematodes, improving formulation and application techniques to enhance bacterial colonization of the root surface, and to identify their mode-of-action. Biotechnological enhancement of the rhizosphere bacteria or transfer of genes responsible for the negative effect on nematodes to other organisms or plants may become possible.

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