

Seed treatment with antagonistic rhizobacteria for the suppression of *Heterodera schachtii* early root infection of sugar beet⁽¹⁾

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SUMMARY

Bacteria isolated from the rhizosphere of sugar beet were used as a seed treatment to suppress *Heterodera schachtii* early root infection. Eight of 290 isolates screened for activity in non-sterilized field soil in greenhouse tests were antagonistic to *H. schachtii*. The eight active isolates were characterized as Gram-negative, motile rods. Three of the eight isolates were identified as *Pseudomonas fluorescens* Migula. All isolates that were tested in the first field test caused a reduction in nematode early root infection levels. One isolate reduced penetration significantly by 75 % and three isolates caused significant increases in yield. In the second year, one isolate significantly reduced nematode penetration. However, none of the bacterial treatments affected plant growth or yield. In greenhouse tests, antagonistic activity was influenced by bacterial density on the seed as well as soil moisture conditions. Antagonistic activity was probably caused by bacterial alteration of root exudates that influenced nematode hatch, attraction, and penetration behavior.

RÉSUMÉ

Traitement des semences avec des bactéries antagonistes pour supprimer l'infestation primaire des racines de betterave à sucre par Heterodera schachtii

Des bactéries isolées de la rhizosphère de betterave à sucre sont utilisées dans un traitement des semences de betterave à sucre en vue de supprimer l'infestation primaire des racines par *Heterodera schachtii*. Huit des 290 isolats testés en serre, dans du sol naturel non stérilisé, étaient antagonistes de *H. schachtii*. Ces huit isolats sont caractérisés par des bactéries mobiles, bacilliformes et gram-négatives; trois de ceux-ci ont été identifiés à *Pseudomonas fluorescens* Migula. Tous les isolats testés lors d'un premier essai au champ provoquent une diminution du niveau de l'infestation primaire des racines. Un des isolats réduit significativement la pénétration de 75 % et trois autres provoquent un accroissement significatif de la récolte. La deuxième année, un isolat a réduit significativement la pénétration du nématode. Cependant, aucun des traitements bactériens n'a d'effet sur la croissance de la plante ou la récolte. Lors d'essais en serre, l'activité antagoniste s'est révélée influencée par la densité des bactéries sur les semences et par les conditions d'humidité du sol. L'activité antagoniste des bactéries est probablement causée par une altération des exsudats radiculaires influençant ainsi le comportement du nématode en ce qui concerne l'éclosion, l'attraction et la pénétration dans les racines.

Effective biological control of plant parasitic nematodes with fungal and bacterial parasites occurs in nature. However, the use of these agents in practical biological control systems has been limited because of lack of specificity, obligate parasitism, energy source requirements, poor establishment in the soil, and inadequate nematode control.

Bacterial seed treatments have been used to control fungal and bacterial root pathogens (Cook & Baker, 1983). We have demonstrated that in greenhouse studies

rhizobacteria applied as a seed dressing can reduce nematode penetration of the root system of potato and sugar beet (Racke & Sikora, 1985; Oostendorp & Sikora, 1986).

The advantages of a seed treatment with rhizobacteria in a biological control system are : 1) their saprophytic nutritional status makes large scale production feasible, 2) only small amounts of inoculum are required, 3) application is simple, 4) independence from energy sources for survival, 5) systemic spread along the sur-

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face of the developing root system, and 6) antagonistic activity on the root surface during the economically important phase of early root infection by the nematode. The reintroduction of an antagonistic organism, isolated from the ecosystem, at low inoculum levels may be environmentally and economically acceptable when compared to the presently available nematicides.

The *Heterodera schachtii*-*Beta vulgaris* model was selected because : 1) early root infection (infection during the first six weeks after planting) is mainly responsible for yield loss, 2) only bacteria with short term antagonistic activity are needed, and 3) *H. schachtii* hatch, attraction, host recognition, and penetration are influenced by root exudates or the root surface. These factors increase the probability that a rhizobacterial seed treatment with subsequent colonization of the rhizosphere may induce antagonistic activity.

The results presented here have been published previously in abstract form (Oostendorp & Sikora, 1986).

Materials and methods

RHIZOBACTERIA ISOLATION

Bacteria were isolated from the rhizosphere of sugar beet collected from the Institut für Pflanzenkrankheiten's experimental field at Bonn-Poppelsdorf, Germany. Beet roots were gently washed free of adhering soil, placed in 10 ml of 0.1 mol/l $MgSO_4$ and the bacteria then separated from the roots by vigorous shaking. Some suspensions were heated to 90° for 10 min for the isolation of sporeforming bacteria. The suspensions were diluted to 10^{-8} and two 0.1 ml aliquots of each dilution were streaked onto Standard I-Agar (Merck), Tryptic-Soy-Agar (Difco) or King's B-Agar (King, Ward & Raney, 1954) at pH values of 5.6 and 7.2. Pure cultures were established and each isolate was tested in the greenhouse for antagonistic activity as described in the following section. Bacterial inoculum for greenhouse trials was produced on solid media, removed from the agar surface with a sterile needle, and the bacteria were suspended in sterile 0.1 mol/l $MgSO_4$. Bacteria for field trials were cultivated in liquid media, concentrated by centrifugation at 5 000 g, washed, and suspended in 0.1 mol/L $MgSO_4$.

SCREENING OF ISOLATES

Isolates were screened for activity in the greenhouse by dipping seeds for 15-30 min into bacterial suspensions adjusted at 560 nm to an optical density (OD) of 1.7 in the first screen and to 2.2 in subsequent screens. Control seeds were treated with 0.1 mol/L $MgSO_4$ without bacteria. Seeds of *B. vulgaris* cv. Kawevera (KWS Seed Production Company, FRG) were used in all tests.

All tests were conducted in unsterilized clay-silt soil with 1.7 % humus collected from a *H. schachtii*-free sugar beet field and mixed 1 : 1 with sand.

In the first screening 290 isolates were tested (two replications each). Isolates demonstrating antagonism were retested in a second screening with five replications each. Nematode inoculum density was adjusted to 1 000 eggs and juveniles/100 ml of soil by mixing cysts of known egg and juvenile contents into the soil. The tests were conducted in a greenhouse at 18°. Both tests were terminated 3-4 weeks after planting. Roots were then washed free of soil, weighed, stained in acid fuchsin, and homogenized in a Waring blender for 10-20 seconds in water. The number of *H. schachtii* juveniles that had penetrated the root system was counted.

DENSITY OF RHIZOBACTERIA INOCULUM ON THE SEED

Optimum inoculum density was determined by dipping seeds into bacterial suspensions of increasing optical densities (Tab. 1). Optimum number per seed was determined by observing plant growth reactions and nematode penetration levels, using the techniques described for the screen. Bacterial colony forming units (cfu) were determined by washing 10 seeds after dipping and then counting the cfu/seed by dilution plating.

INFLUENCE OF SOIL MOISTURE

The importance of moisture for antagonistic activity was studied in the greenhouse. Using the same soil mix as in the screening experiment, isolates A-59 and A-57 were tested at three inoculum densities and at two soil moisture levels, 40 and 80 % of water capacity. Soil moisture was maintained by daily addition of the appropriate amount of water following determination of field capacity by gravimetric techniques.

FIELD TRIALS

Trials were conducted in a *H. schachtii*-infested field on the Institut's experimental test site in Bonn-Poppelsdorf. Nematode P_i levels were determined independently for each plot. Treatment replicates were distributed among the different plots so that mean P_i densities in each treatment were similar. Plot size was 2.5 × 4 m in 1984, and 2.5 m × 6 m in 1985. The isolates tested in 1984 and the inoculum densities are given in Table 2. In 1985, the isolates A-57, A-59, P-510, and P-523 were tested again at the same inoculum densities. Bacteria treated seed of the beet cultivar Kawevera were planted at a density of 10 seeds/m row with five rows per plot in both years. Each treatment was replicated five times in 1984 and six times in 1985. The nematicide Temik 5G at 1 g/m row (1 kg a.i./ha) was used to compare the level of bacterial antagonistic activity with standard techniques of nematode control. The control seed was treated with 0.1 mol/l $MgSO_4$.

Table 1
Influence of eight selected rhizobacteria isolates on plant growth
and *H. schachtii*-penetration at different inoculum densities

Strain	Inoculum density OD	cfu/seed	Plant weight in % Control = 100	Penetration in % Control = 100
A-3	1.7	9.5×10^6	119	31**
	2.1	2.2×10^7	88	56
	2.5	6.2×10^7	82	60
A-57	1.8	3.1×10^7	103	67
	2.3	9.0×10^7	97	63*
	2.7	1.1×10^8	83	43**
A-59	2.0	4.0×10^7	117	58*
	2.4	7.1×10^7	111	41**
	2.7	1.1×10^8	90	71
T-58	1.7	1.7×10^7	157	60
	2.3	4.6×10^7	173	37*
	2.8	6.1×10^7	180	70
P-510	1.7	5.2×10^7	147	35**
	2.2	9.8×10^8	103	34**
	2.7	3.3×10^9	133	15**
P-523	1.8	1.7×10^7	149	36**
	2.3	3.0×10^7	129	32**
	2.8	4.2×10^8	123	67
P-76	1.5	1.1×10^7	118	53
	2.1	1.9×10^7	129	39*
	2.5	3.3×10^7	137	43*
P-741	1.5	2.3×10^6	88	61
	2.2	1.2×10^7	107	31**
	2.9	6.4×10^7	132	21***

Significant differences from the control according to the LSD test :

* p = 5 %

** p = 1 %

***p = 0.1 %

Table 2
Inoculum density of rhizobacteria used
in field test given as optical density
(OD) of bacterial suspension.

Isolate	OD	Isolate	OD
A-3	1.7	P-510	2.7
A-57	2.7	P-523	2.3
A-59	2.4	P-76	2.3
T-58	2.3	P-741	2.9

Early root infection was measured by randomly selecting 10 seedlings from each plot at 48 and 37 days after sowing in 1984 and 1985, respectively. The seedlings were washed free of soil and weighed. The roots

were stained and nematode root penetration levels determined as already described. Total yield was estimated after 192 days in 1984 and 169 days in 1985. The beets from the core area of each plot (7 m² and 12 m² in 1984 and 1985, respectively) were weighed after removal of adhering soil.

Results

SCREENING OF RHIZOBACTERIA FOR ACTIVITY

Twenty-one of the 290 rhizobacteria isolates tested in the greenhouse reduced nematode early root infection and fifteen isolates increased nematode infection 3-4 weeks after sowing in the first screening. However, only eight isolates reduced penetration in the second screen. All antagonistic isolates were Gram-negative,

motile rods. They showed no anaerobic growth and produced fluorescent pigments on King's B medium, except of A-59 and P-510. Three of the eight isolates (T-58, A-59, P-523) were identified as *Pseudomonas fluorescens* (Trevisan) Migula.

RHIZOBACTERIA INOCULUM DENSITY

The eight isolates, antagonistic to *H. schachtii*, were tested at increasing inoculum densities (Tab. 1). The number of cfu per seed tested ranged between 2.3×10^6 and 3.3×10^9 . Antagonistic activity increased with increasing density with isolates A-57, P-510, P-76, and P-741, whereas A-3 exhibited reduced activity at higher inoculum densities. Conversely, A-59, T-58, and P-523 were most effective in reducing early root infection at moderate densities.

SOIL MOISTURE

Antagonistic activity of isolate A-59 differed slightly at the two moisture levels tested (Fig. 1 A). The level of antagonism of isolate A-57, however, varied with soil moisture level (Fig. 1 B). At the highest cfu/seed density, isolate A-57 caused an 80 % reduction in nematode penetration in dry soil when compared to the untreated control. At the same density in wet soil, no significant reduction in penetration was detected. Plant fresh weight was not influenced by either bacterial strain (Fig. 2 A & B).

FIELD TRIALS

Seed treatment with seven of the eight rhizobacteria isolates caused a slight increase in plant weight 48 days after sowing in 1984 (Fig. 3 A). Nematode penetration decreased in all treatments when compared to the untreated control. *P. fluorescens* isolate A-59 caused a significant 75 % reduction in *H. schachtii* penetration when compared to the control (Fig. 3 B). Temik caused a 90 % reduction in early root infection. Isolates A-57, P-510, P-523 as well as Temik induced significant increases in sugar beet yield (Fig. 3 C).

In 1985, isolates A-57, A-59, P-510, and P-523 suppressed nematode early root infection when compared to the control (Fig. 4 B). Strain P-523 and Temik caused significant reductions in *H. schachtii* early root infection. Plant fresh weight and yield, however, were not significantly affected (Fig. 4 A & C).

Discussion

In the initial screening, 21 of 290 rhizobacterial isolates (7.2 % of those tested) were antagonistic to *H. schachtii*. However, the number of isolates exhibiting consistent antagonistic activity decreased to eight (2.8 %) after additional screening. The number of effective

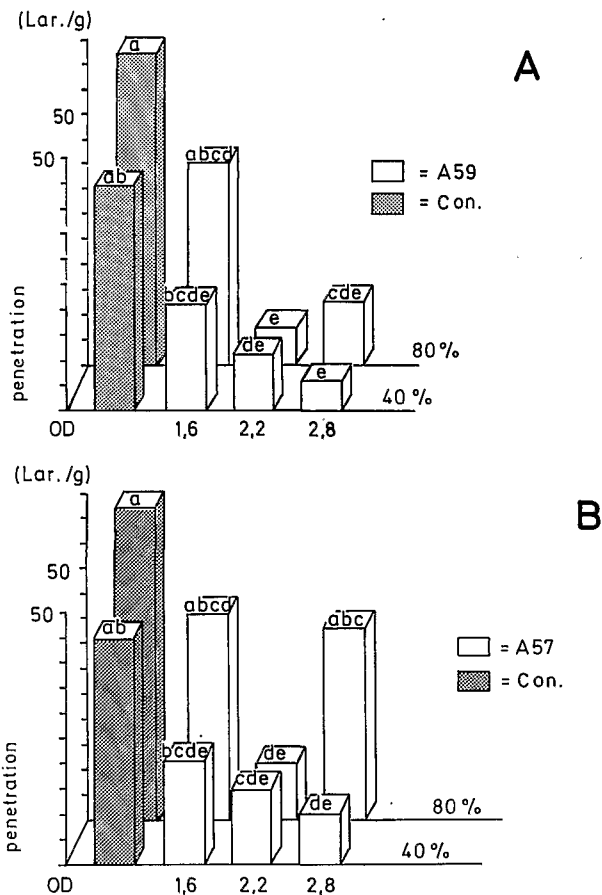


Fig. 1. Influence of rhizobacteria seed treatment with isolate A-59 (A) and A-57 (B) on *Heterodera schachtii* early root penetration of sugar beet at different inoculum densities (OD) and soil moisture levels of 40 % and 80 % of water capacity 26 days after planting. Columns with different letters are significantly different ($P = 5\%$) according to the Duncan's Multiple Range Test. The analysis for penetration was conducted following Log-transformation.

isolates was lower than that reported by Zavaleta-Meija and Van Gundy (1982) who found 12 % of the bacterial isolates tested active against *Meloidogyne*.

Plant growth promoting activity was detected in 1 % to 4 % of the bacteria isolated by Suslow *et al.* (1979). The results of this and previous studies demonstrate the existence of rhizobacteria in the soil which possess either antagonistic activity to soilborne plant pathogens or plant growth promoting characteristics.

That some rhizobacteria increase nematode infection levels is also of significant importance. Similar results with *Globodera pallida* (Stone, 1973) Behrens, 1975 have been reported (Racke & Sikora, 1985).

Although we did not test the rhizobacteria for activity against other soilborne pathogens, multiple effects on

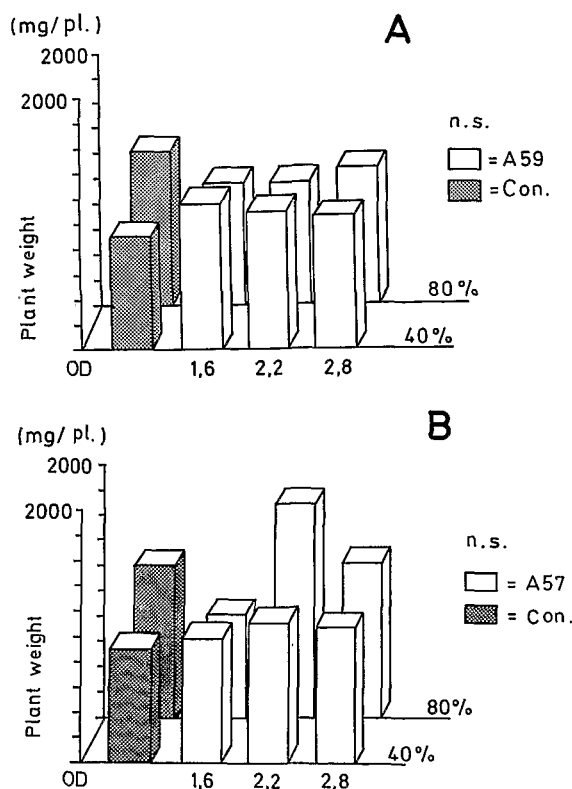


Fig. 2. Influence of rhizobacteria seed treatment with isolates A-59 (A) and A-57 (B) on sugar beet plant fresh weight in mg at different inoculum densities (OD) and soil moisture levels of 40 % and 80 % of water capacity 26 days after planting. The differences were not statistically significant = n.s.

other soil organisms have been shown to exist (Racke & Sikora, 1986). Rhizobacteria, antagonistic to *G. pallida* on potato, increased *Erwinia carotovora* (Jones) Bergey *et al.* soft rot incidence.

Bacterial isolates that reduced *H. schachtii* early root infection were all Gram-negative rods, and three of eight were pseudomonads. Pseudomonads have previously been used as seed treatments for plant growth promotion and biological control of bacterial or fungal pathogens (Cook & Baker, 1983). Their versatile metabolism, fast growth, active movement, and ability to readily colonize the root surface make these rhizobacteria especially suitable for seed bacterization.

Siderophore and antibiotic production by fluorescent pseudomonads is often proposed as a mechanism of action in reduction of soilborne plant diseases or in plant growth stimulation (Howell & Stipanovic, 1979, 1980; Klopper & Schroth, 1981; Misaghi *et al.* (1982)). Nematode activity, however, is probably not affected by siderophore induced iron deficiency in the rhizosphere. We believe that the antagonistic reactions observed in our studies may have been based on mechanisms that altered hatch, attraction, or host recognition.

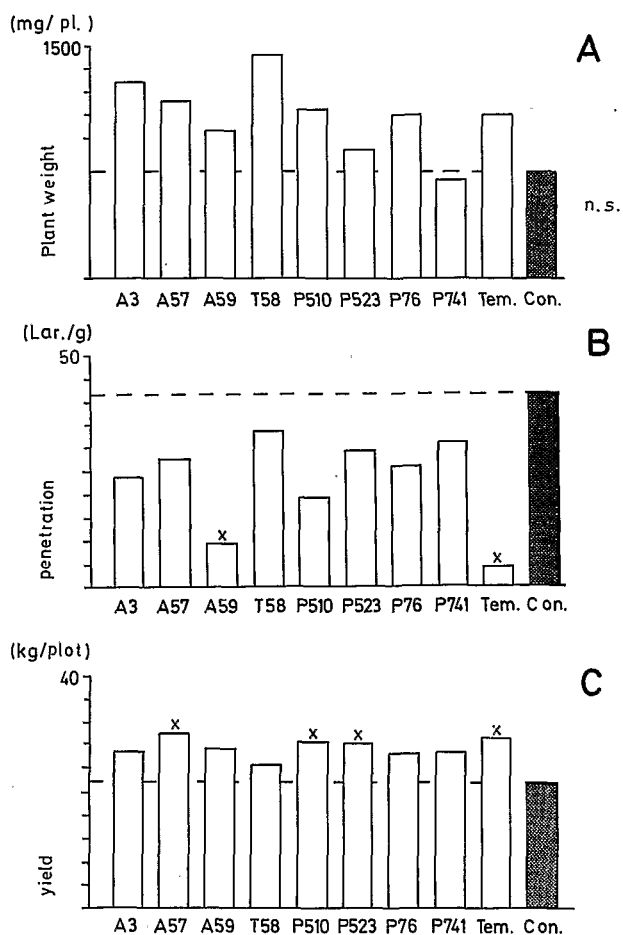


Fig. 3. Influence of eight rhizobacteria isolates, used as sugar beet seed treatments, on plant weight (A) and *Heterodera schachtii* early root penetration (B) 48 days after planting and on total yield (C) in a 1984 field trial. Columns marked with « x » are significantly different from the control ($P = 5\%$) according to the Duncan's Multiple Range Test. Penetration (B) was calculated following a square root transformation.

The inoculum density was an important factor governing the efficacy of the bacterial treatment. Isolates A-3, A-59, T-58, and P-523 showed reduced antagonistic activity at high inoculum levels (Tab. 1). This effect was not found with A-59 in the second trial at different moisture levels (Fig. 1 A). Isolate A-57 which was most effective at high inoculum densities in the first trial showed reduced activity under high moisture conditions when applied at high densities (Fig. 1 B). Decreased activity of antagonists at high inoculum levels has been reported (Lynch, 1978; Schneider, 1984; Brown, Kepner & Smart, 1985). Brown, Burlingham & Jackson (1964) reported plant growth promoting activity of *Azotobacter* at 60 % water holding capacity, but inhibitory effects at

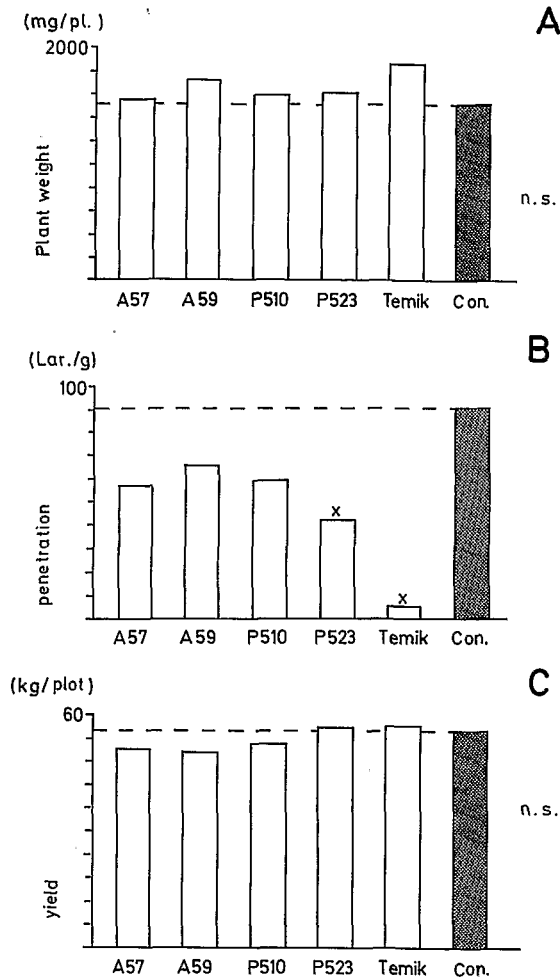


Fig. 4. Influence of four rhizobacteria isolates, used as sugar beet seed treatments, on plant weight (A) and *Heterodera schachtii* early root penetration (B) 37 days after planting and on total yield (C) in a 1985 field trial.

Columns marked with « x » are significantly different from the control ($P = 5\%$) according to the Duncan's Multiple Range Test. Penetration (B) was calculated following a square root transformation.

100 % water holding capacity. In some cases, high bacterial inoculum densities may be less effective in reducing nematode penetration because of inadequate oxygen levels on the root or seed surface.

The results showed that under field conditions rhizobacteria can significantly suppress *H. schachtii* early root infection. The level of antagonism was in most cases lower than that obtained in the greenhouse. The differences in the level of antagonism in the field between the two years were probably caused by variation in environmental conditions. In 1984, soil moisture was low the first two weeks after sowing, whereas in 1985, heavy

rainfall produced high soil moisture levels after sowing. The greenhouse studies demonstrated that isolates exist that can withstand both low and high moisture levels.

The microbial flora of the soil is another important factor to be considered. Although all isolates used in this study were isolated from the field that was used in the field experiments, they were selected for activity in a different soil. The competition of the microbial soil flora may reduce the activity of the inoculum under field condition or in other soils. Furthermore, changes in the environmental conditions do not only influence the inoculated bacteria, but may favor or be unfavorable for competitors in the soil.

More detailed studies are needed on the composition of the rhizosphere population, the effect of cultivar on bacterial population dynamics, the influence of inoculum density on antagonistic activity, the survival of the inoculum under adverse conditions, and the role that environmental conditions play in altering the activity of rhizobacteria.

An attempt to overcome problems of varying efficacy may be attained by strain mixing, improved inoculation techniques, or gene transfer of the active genetic source of antagonism to the host plant.

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