

Greenhouse studies comparing strains of the fungus *Verticillium lecanii* for activity against the nematode *Heterodera glycines*

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Summary – In a greenhouse experiment, five strains of the fungus *Verticillium lecanii* were compared for their effects on soybean cyst nematode population densities. Four strains were mutants selected for increased tolerance to benomyl; the fifth strain was a wild type (ATCC 58909). Alginate prills containing fungus were applied at 0.5 g and 5.0 g prills per pot (each pot contained 530 g soil mixture). At the 5.0 g prills per pot application rate, all four mutant strains caused significant reductions in cyst numbers compared to controls without fungus. No strains were effective against the nematode when the prills were applied at 0.5 g per pot.

Résumé – Études comparatives en serre du champignon *Verticillium lecanii* pour son activité contre le nématode *Heterodera glycines* – Lors d'une expérimentation en serre, cinq souches du champignon *Verticillium lecanii* ont été comparées pour leurs effets sur les densités de population du nématode à kyste du soja. Quatre de ces souches étaient des mutants sélectionnés pour l'augmentation de la tolérance au benomyl; la cinquième souche était une souche sauvage (ATCC 58909). Des billes d'alginate renfermant le champignon ont été appliquées au taux de 0,5 et 5,0 g de billes par pot (chaque pot contenant 530 g de sol). Au taux de 5,0 g de billes par pot, les quatre souches mutantes entraînaient des réductions significatives du nombre de kyste par rapport au témoin sans champignon. Aucune souche n'a eu d'effet sur le nématode au taux de 0,5 g par pot.

Key-words : benomyl tolerance, biological control, fungus, *Heterodera glycines*, mutant, nematode, soybean cyst nematode, *Verticillium lecanii*.

The fungus *Verticillium lecanii* (A. Zimmermann) Viégas is an antagonist against some species of plant-parasitic nematodes (Hänssler & Hermanns, 1981; Gintis *et al.*, 1983; Hänssler, 1990). One isolate of *V. lecanii* significantly decreased percentages of live *Heterodera glycines* Ichnohe eggs in a Petri dish assay (Meyer *et al.*, 1990). This decrease in egg viability occurred without a concomitant colonization of live eggs, indicating that the fungus acted through production of deleterious compounds. Conidia of that strain were subsequently irradiated with ultraviolet light (Meyer, 1992), and four resulting mutant strains were selected for increased tolerance to the fungicide benomyl. In a previous greenhouse study, the efficacy of the wild type strain against *H. glycines* was compared with that of mutant strain M2S1 (Meyer & Meyer, 1995). The two strains were tested in different soil types, and at various nematode and fungus application rates. In some of those tests, the mutant strain was more efficacious than the wild type strain at reducing *H. glycines* population densities (Meyer & Meyer, 1995). The objective of the current study was to test the activity of all four benomyl mutants against *H. glycines* in greenhouse pots, and to

identify the most efficacious strain. Because the wild type strain did not decrease *H. glycines* population levels at a high nematode application rate (Meyer & Meyer, 1995), a similar inoculum level was applied. This was done to determine whether all of the mutant strains would show activity at nematode population levels where the wild type strain was ineffective. Additionally, in the current study, the efficacy of all five strains was determined after the first (35 days) and second (65 days) nematode generations.

Materials and methods

The *V. lecanii* isolates used were the wild type strain (American Type Culture Collection 58909) and the mutant strains deposited as Agricultural Research Service Culture Collection, NRRL 18725, 18726, 18727, and 18728 (Meyer, 1992), equivalent to Beltsville Nematology Laboratory strains M1S1, M2S1, M9S1, and M10S1, respectively. The strains were maintained on potato dextrose agar (PDA), transferred periodically, and stored in a refrigerator (4 °C). For inoculation of greenhouse pots, the fungi were grown in Erlenmeyer

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flasks on orbital shakers at 25 °C and harvested by centrifugation (Meyer, 1994). Alginate prills (Fravel *et al.*, 1985) were made with 5 g wheat bran (ground to a particle size of less than 1 mm), 15 g alginate, and 100 g homogenized wet fungus (*ca.* 2.52-6.88 g dry fungus) per l of water (Meyer, 1994). Prills were *ca.* 10-20 % dry weight fungus. *Heterodera glycines* race 3 for use as inoculum was grown in the greenhouse on the susceptible soybean *Glycine max* (L.) Merr. cv. Essex. Three to 4 months after inoculation of nematodes onto soybean seedlings, cysts were collected by a modified centrifugal-flotation technique (Meyer & Meyer, 1995), and the eggs were extracted by grinding the cysts in a manual homogenizer. Eggs and second-stage juveniles (J2) were then used as inoculum for experiments.

Soybean seeds (cv. Essex) were sown into styrofoam flats containing sterile sand and were transplanted 1-2 weeks later into an unsterile soil mixture made of sand and compost (compost was 16 m³ top soil : 8.4 m³ manure, mostly from dairy cattle : 36 kg 5-10-5 NPK fertilizer : 109 kg high magnesium dolomitic lime). Final composition of the sand/compost soil mixture was 96 % sand, 1 % silt, 3 % clay, 0.4 % organic matter; pH 7.2. Experimental units were 10-cm-diameter pots (538 cm³ volume) each containing one plant in *ca.* 530 g soil mixture.

On the day of transplanting, a hole (4-5 cm deep) was made 2-3 cm from each root system, and an aqueous suspension of 8000 eggs and J2 was poured into each pot. The nematode inoculation rate was selected because a previous study (Meyer & Meyer, 1995) found that *V. lecanii* strains were less effective against high *H. glycines* populations (10 000 nematodes per pot) than against low populations (300 nematodes per pot). Therefore, larger numbers of nematodes were added to ensure that only the most efficacious strains would be identified.

Following inoculation with nematodes, prills were placed into each hole, and the holes were filled in with soil mixture. Each fungus strain was applied at two rates : 5.0 and 0.5 g prills per pot (*ca.* 0.9 and 0.09 % prill dry weight per soil mixture dry weight, respectively). Prills without viable fungus were found not to affect *H. glycines* population densities (Meyer & Meyer, 1995), so control pots were inoculated with nematodes only. There were 20 pots per treatment for each repetition of the experiment. Half of the pots per treatment were harvested 35 days after inoculation, and the remaining ten pots per treatment after 65 days. Pots were arranged in randomized complete blocks, with the 35-day and 65-day harvest groups laid out separately from each other. This entire procedure was conducted twice : a first experiment followed by a second, replicate experiment. Greenhouse temperatures were maintained near 27 °C (16-43 °C range). Supplemental lighting (400 W, high pressure sodium bulbs) provided 12-16 h daylight per 24 h period. At harvest, total numbers of cysts per-

pot were collected and counted on lined filter papers (Krusberg *et al.*, 1994). Cysts were counted as indicators of the population level because of the mode of action of the fungus. *V. lecanii* appears to decrease egg viability by production of deleterious compounds, rather than by parasitism. Because uncolonized dead eggs are difficult to distinguish from live eggs, egg counts from greenhouse pots would not accurately represent viable egg numbers.

Following the second repetition of the experiment, soil mixture from each pot was suspended in water at a ratio of 0.02 g/ml and plated onto agar media in Petri dishes to test for live *V. lecanii* (Meyer & Meyer, 1995). The media selected for this study were those that provided the best isolation results in prior experiments. Media used were : *i*) Ausher's medium N° 2 (Ausher *et al.*, 1975) modified by replacement of PCNB with benomyl (for controls and pots treated with wild type strain); and *ii*) PDA amended with antibiotics, benlate, and ethanol (PDA ABE : Meyer, 1994). For the control pots, PDA ABE was amended with 0.2 g benlate/l (PDA ABE 100). Soil mixture from pots treated with each mutant was plated onto PDA ABE 5 (0.01 g benlate/l medium), PDA ABE 100, and PDA ABE 1000 (2.0 g benlate/l). Soil mixture from each pot was plated onto two Petri dishes of each medium.

The number of cysts per pot data was analyzed for each harvest time as a two factor design using PROC MIXED in SAS/STAT 6.11 (SAS Institute, 1996). Experiment was the random factor while fungus treatment was the fixed factor; *n* = 17-20 for each strain application rate at each harvest time. Combined data from the two harvest times was analyzed as a three-factor design using SAS 6.08 PROC MIXED (SAS Institute, 1989). The experiment repetition was treated as a random factor, whereas days to harvest and fungus treatment were fixed factors; *n* = 36-40 for each strain application rate.

To correct for variance heterogeneity, the natural log (ln) transformation was used for all analyses. The number of cysts per pot is reported as least squares means (ls means).

Results and discussion

Treatment was highly significant for each harvest time. For the 35 day harvest time, *F* = 8.95 with *P* = 0.0001. For the 65 day harvest time, *F* = 4.66 with *P* = 0.0001. When the 35 and 65 day harvests were combined, analysis indicated that there was no significant day effect (*P* = 0.5850) or treatment x day interaction (*P* = 0.5215). The treatment effect was significant (*P* = 0.0001).

We anticipated that the fungi would take some time to proliferate in the soil. Consequently, it was hypothesized that *V. lecanii* would not affect the nematode inoculum, but instead would kill eggs produced by first-generation females on the soybean roots. If true, the cyst numbers at the 35 day harvest should have been similar in the

control and fungus-treated pots. However, the results indicated that several effective strains were deleterious to eggs that did not hatch immediately, or were pathogenic to juveniles or to young females. Strains M1S1, M2S1, and M9S1, when applied at 5.0 g prills per pot, all significantly reduced cyst numbers ($P < 0.05$) when compared to untreated controls (Table 1). Reductions in cyst numbers compared to the controls were 53, 60, and 44 %, respectively. At the 35-day harvest time, treatment with each fungus strain at the 5.0 g prill application rate also resulted in fewer cysts than treatment with the lower application rate (Table 1). For most strains, the difference was significant at $P < 0.05$; $P < 0.10$ for one strain (Table 1). Reductions were 34, 53, 62, 43, and 26 %, respectively, with the wild type strain and strains M1S1, M2S1, M9S1, and M10S1.

At the 65 day harvest time, strains M1S1 and M2S1 applied at 5.0 g prills per pot again reduced cyst numbers compared to controls ($P < 0.05$, Table 1). Reductions were 41 and 44 %, respectively. While strain M9S1 was not effective, application of strain M10S1 resulted in a 42 % reduction in cyst numbers ($P < 0.05$). All four of the mutant strains showed significant differences in cyst numbers when higher and lower application rates were compared within a strain ($P < 0.05$ or $P < 0.10$; Table 1). Cyst number reductions at the 5.0 g prill application rate, when compared to the 0.5 g application rate, were 56, 56, 34, and 32 % respectively, with strains M1S1, M2S1, M9S1, and M10S1.

Because of the correlation between 35 and 65 day results, data for the two harvest dates from both trials were combined for each treatment and presented as an

overall mean (Table 1). Combining data from both harvest times to indicate overall efficacy of each strain provided similar results to the analyses of individual harvest times. Treatments that resulted in cyst number reductions compared to controls ($P < 0.05$) were strain M1S1 (46 % reduction) and M2S1 (57 % reduction), both applied at 5.0 g prills per pot. These strains had significantly reduced cyst numbers at both the 35- and 65-day harvest times. Smaller cyst number reductions ($P < 0.10$) at the 5.0 g prill application rate were also recorded for strains M9S1 (29 % reduction) and M10S1 (30 % reduction). These strains had each been effective at one harvest time. Application of any mutant strain at 0.5 g prills per pot or of the wild type strain at either rate did not reduce the numbers of cysts per pot.

The 0.5 g prill application rate was compared with the higher application rate for each treatment in the combined analysis (Table 1). With strains M1S1, M2S1, and M9S1, the 5.0 g application rate resulted in fewer cysts ($P < 0.05$) than the 0.5 g application rate (54, 63, and 38 % reductions, respectively). The wild type strain (31 % reduction compared to lower application rate) and strain M10S1 (29 % reduction) also showed some difference between the two application rates ($P < 0.10$).

The results of this research agreed with an earlier study in which the wild type strain of *V. lecanii* reduced *H. glycines* population densities when the nematode was added at 300 eggs/J2 per pot, but not at 10 000 per pot (Meyer & Meyer, 1995). In the current investigation, with 8 000 eggs/J2 added to each pot, the wild type strain did not decrease cyst numbers, indicating that this strain needs a lower nematode population to be effective.

Table 1. Number of *Heterodera glycines* cysts per pot (reported as least squares means) after treatment with the fungus *Verticillium lecanii*. Five *V. lecanii* strains were tested individually at 0.5 g and 5.0 g prills per pot. Results are from two trials of the experiment, with 35-day and 65-day harvest times presented separately and combined.

Treatment	Day 35			Day 65			Days 35 and 65 combined		
	Mean	<i>P</i> value vs control	<i>P</i> value of 0.5 vs 5.0	Mean	<i>P</i> value vs control	<i>P</i> value of 0.5 vs 5.0	Mean	<i>P</i> value vs control	<i>P</i> value of 0.5 vs 5.0
Control	312	—	—	255	—	—	278	—	—
Wild type 0.5	366	0.3487	—	338	0.2288	—	350	0.2347	—
Wild type 5.0	240	0.1233	0.0125**	245	0.8596	0.1569	242	0.4799	0.0557
M1S1 0.5	309	0.9527	—	347	0.1880	—	327	0.4033	—
M1S1 5.0	146*	0.0001	0.0001**	151*	0.0221	0.0003**	149*	0.0014	0.0000**
M2S1 0.5	331	0.7296	—	320	0.3403	—	327	0.4080	—
M2S1 5.0	126*	0.0001	0.0001**	142*	0.0114	0.0005**	120*	0.0000	0.0000**
M9S1 0.5	306	0.9130	—	333	0.2551	—	317	0.5024	—
M9S1 5.0	174*	0.0008	0.0010**	221	0.5373	0.0733	197*	0.0776	0.0141**
M10S1 0.5	339	0.6390	—	218	0.4902	—	272	0.9215	—
M10S1 5.0	251	0.2039	0.0815	149*	0.0192	0.0885	194*	0.0636	0.0762

* Mean value significantly lower than control; $P < 0.05$. Exceptions: for strains M9S1 and M10S1, days 35 and 65 combined, $P < 0.10$.

** Cyst numbers counted after application of the higher rate of fungus significantly different ($P < 0.05$) from cyst numbers counted after application of fungus at the lower rate. The values in these columns not marked with asterisks were significant at $P < 0.10$, with the exception of the wild type strain harvested at day 65.

Table 2. Isolation of *Verticillium lecanii* from sand.

Treatment-Fungus strain and grams prills/pot	Harvest period	Number of pots from which each fungus was isolated*	Media on which each fungus was isolated**
Control	35 days	0	0
	65 days	0	0
Wild type 0.5	35 days	1	Ausher's (2)
	65 days	0	0
Wild type 5.0	35 days	0	0
	65 days	0	0
M1S1 0.5	35 days	2	PDA ABE 100 (1), 1000 (4)
	65 days	2	PDA ABE 100 (1), 1000 (3)
M1S1 5.0	35 days	3	PDA ABE 1000 (7)
	65 days	6	PDA ABE 100 (2), 1000 (9)
M2S1 0.5	35 days	5	PDA ABE 5 (2), 1000 (8)
	65 days	3	PDA ABE 1000 (5)
M2S1 5.0	35 days	1	PDA ABE 1000 (2)
	65 days	6	PDA ABE 1000 (8)
M9S1 0.5	35 days	0	0
	65 days	3	PDA ABE 100 (3), 1000 (4)
M9S1 5.0	35 days	3	PDA ABE 100 (2), 1000 (6)
	65 days	6	PDA ABE 100 (2), 1000 (8)
M10S1 0.5	35 days	1	PDA ABE 1000 (2)
	65 days	1	PDA ABE 1000 (1)
M10S1 5.0	35 days	0	0
	65 days	0	0

* Soil was taken from ten pots per treatment at each harvest period of trial 2. Exceptions from the 65-day harvest: M2S1 0.5 g, eight pots; M9S1 5.0 g and M10S1 0.5 g, nine pots.

** Values in parentheses are the number of Petri dishes from which the fungus was isolated. For each harvest period, sand was plated onto four Petri dishes total per control pot, two per wild type treatment pot, and 6 per mutant strain treatment pot.

tive. The mutant strain (M2S1) that reduced *H. glycines* population densities in the earlier study was also effective in the present study, and additional mutant strains demonstrated activity against *H. glycines*. Long-term maintenance of the fungus strains in the laboratory did not result in loss of biocontrol activity against *H. glycines*. As in the prior investigation, higher application rates of the fungus strains were more effective than lower fungus application rates.

All five *V. lecanii* strains were recovered from the soil mixture of treated pots (Table 2). PDA ABE 1000 was the most effective medium for isolation of the mutant strains. There was a tendency to recover the mutant fungus strains from more pots at the 65 day harvest than at the 35 day harvest. This may indicate that the mutant strains proliferated further into the soil during the longer time period, and were not just surviving statically in the prills. In contrast to the mutant strains, the wild type strain was only isolated from one pot, harvested at 35 days. These data support the hypothesis that the wild

type strain was relatively ineffective against nematodes in this study because it did not compete as well in the soil as did some of the mutant strains.

The results of this study indicate that certain mutant strains of *V. lecanii* demonstrated enhanced activity against *H. glycines* and increased persistence in the soil. These efficacious strains merit further study to determine whether they would provide economical biocontrol agents for *H. glycines* on soybean.

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