Interactions Among Selected Endoparasitic Nematodes and Three Pseudomonads on Alfalfa[1]

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Abstract: Meloidogyne hapla, Pratylenchus penetrans, and *Helicotylenchus dihystera,* reduced the growth of 'Saranac AR alfalfa seedlings when applied at concentrations of 50 nematodes per plant. All except *P. penetrans* reduced seedling growth when applied at 25 per seedling. *M. hapla* reduced growth when applied at 12 per seedling. Nematodes interacted with three pseudomonads to produce greater growth reductions than were obtained with single pathogens, suggesting synergistic relationships. *Ditylenchus dipsaci,* applied at 25 or 50 nematodes per seedling, reduced plant weight compared with weights of control plants, but did not interact with test bacteria. All of the nematodes except *D. dipsaci* produced root wounds which were invaded by bacteria. *Key words: Medicago sativa* L., *Meloidogyne hapla, Pratylenchus penetrans, Ditylenchus dipsaci, Helicotylenchus dihystera, Pseudomonas viridiflava, Pseudomonas corrugata, Pseudomonas marginalis.*

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Several species of fungi cause crown and root rots of alfalfa. Many rot diseases, however, cannot be ascribed to the activities of a single pathogen and generally are attributed to the effects of disease complexes (6). The nature of the crown and root rot complex of alfalfa is poorly understood, but it is believed to consist of a number of pathogenic fungi interacting with other pathogens and nonpathogenic soil organisms, as well as abiotic influences. Rot organisms other than fungi may be important interactivants which are not detected by isolation procedures that favor the recovery of pathogenic fungi.

This investigation was conducted to determine whether three pseudomonads, isolated from symptomless or discolored alfalfa roots, and which decayed needle-wounded alfalfa roots in vitro (8), could rot roots of alfalfa plants stressed by infection with any of several endoparasitic nematodes that commonly occur in alfalfa field soil in Pennsylvania.

MATERIALS AND METHODS

Seeds of *Medicago sativa* L. 'Saranac AR' were surface-sterilized with a 10-min soak

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in 1.05% NaOCl, followed by a 30-min soak in a 0.02% solution of $HgCl_2$ in 70% ethanol, followed by a thorough rinsing with sterile distilled water.

Nematodes: Populations of *Meloidogyne hapla* (Chitwood) were maintained in a greenhouse on 'Bonny Best' or 'Rutgers' tomato (*Lycopersicon escolentum* L.) plants. Eggs were extracted from roots of infected plants and surface sterilized by the method of Hussey and Barker (4), washed with sterile distilled water, and used immediately.

Surface sterilized *Pratylenchus penetrans* (Filipjev) and *Ditylenchus dipsaci* (Kuhn) (alfalfa race) were maintained on callus tissue of Saranac AR alfalfa grown on Krusberg's medium (5) in the dark at 20 C. Nematodes were extracted from callus by incubating the tissue in sterile water for 3 d on a wrist-type shaker. The water was then passed through a sieve with 44-μm openings to catch the nematodes which were used immediately.

Helicotylenchus dihystera (Cobb) were maintained in the greenhouse on field corn. Nematodes were separated from soil by a modification of the Christie-Perry method and collected by using a fiberglass milk filter. The filter was placed in a Baerman funnel, and nematodes were drawn off and surface sterilized after 12 h. Nematodes were used immediately following sterilization.

Surface sterilization of nematodes: An antibiotic solution containing 25 mg Penicillin G and 10 mg furazolidone was mixed with a solution containing 25 mg a.i. each of tetracycline HCl, streptomycin sulfate, and captan 50W in 20 ml of distilled water, and the volume brought to 50 ml. The mixed antibiotic solution was passed through a 0.22-μm pored membrane filter and used immediately.

Nematodes were collected in 0.02% $HgCl_2$, gently agitated for 15 min, washed with sterile distilled water, transferred to half-strength antibiotic solution for 1 h, washed with sterile distilled water, and diluted to the desired concentrations. Preliminary experiments indicated that the treatment eliminated contaminating organisms and that the motility of treated nematodes apparently was not impaired.

Bacteria: Pseudomonas marginalis (Brown) Stevens (PSU isolate #517) is a common soil organism which discolors alfalfa roots (9). *Pseudomonas viridiflava* (Burkholder 1930) Dowson 1939 is not associated with distinct symptoms on alfalfa roots in the field and produces a distinctive blue intracellular pigment when grown on yeast-dextrose-$CaCO_3$ agar (YDCA). *Pseudomonas corrugata* Roberts & Scarlett (isolate #338), a nonfluorescent strain, was isolated from symptomless roots of greenhouse-grown alfalfa (7).

Preliminary tests indicated that all of these bacteria produced localized necrotic lesions when stab-inoculated into mature roots of 3-month-old Saranac AR alfalfa plants by the procedure of Lukezic (7), but they differed in the type of lesion produced; *P. marginalis* produced smaller, less discolored lesions than did either *P. syringae* or *P. corrugata.*

Bacterial cells for inoculation were grown on YDCA for 18–24 h, washed twice and adjusted to 2×10^8 cells per ml of sterile distilled water.

Plant culture: Transparent plastic storage boxes (41 cm × 27 cm × 10 cm) were filled to a depth of 3 cm with sterilized white quartz sand. Hollow 15-mm-d polyethylene stoppers embedded in the sand served as growing vessels for alfalfa seedlings. After chambers were sterilized with propylene oxide vapor, sterile river-bottom sand was placed in each stopper along with suspensions of the organisms to be studied and the desired numbers of host seeds or seedlings. Prepared chambers were placed in the greenhouse at a temperature of 72 ± 3 C. Sand in the stoppers was moistened, when necessary, with sterile solution #1 of Hoagland and Arnon (3).

Seedlings were grown for 2 wk, after which they were removed from the stoppers, washed free of sand, blotted dry, and weighed. Fresh weights of seedlings were recorded.

Interaction experiments: Treatments applied to each stopper included no pathogens (control), bacteria ($\sim 2 \times 10^8$ cells/ml), nematodes (36, 75, or 150 per ml), or nematodes and bacteria (one species of each, at the concentrations noted above). One ml of inoculum (or sterile water) was

applied to each stopper. Each experiment utilized three disinfested seeds per stopper and 12 stoppers per treatment.

Water-treated stoppers were placed at the corners and center of each chamber. Two weeks after seeding, these stoppers (with contents) were aseptically transferred to flasks containing sterile nutrient broth. If turbidity was observed in a flask after 48 hr, data from the chamber were not analyzed.

The effects of nematode-bacterium interactions on older plants were studies using 2-wk-old aseptically grown seedlings planted five per pot in 10-cm pots containing an aerated-steam pasteurized mixture of soil, peat, and perlite 1:1:1 (1). Ten milliliters of suspensions of single bacterial isolates in sterile tap water ($\sim 2 \times 10^8$ cells/ml) were applied by pipet to holes in the soil in each pot. Each treated pot received suspensions of 5,000 disinfested nematodes of the test species (eggs in the case of *M. hapla*) in 10 ml sterile tap water; control pots received sterile water only. Twenty plants were used per treatment.

Six weeks after soil infestation, the plants were cut at the soil line and fresh weight of above-ground portions determined. The experiment was conducted twice.

Data analysis: Crossed-factors analyses of variance were performed on each replication of each experiment; analyses were then run on pooled data, using pooled variances. Mean separation was performed using Duncan's modified (Bayesian) least significant difference tests (2), with k = 100 (P = 0.05).

RESULTS AND DISCUSSION

Interaction chamber studies: The results of nematode-bacterium interaction studies conducted on seedlings in chambers are shown in Table 1. Only fresh weights of seedlings were recorded; preliminary testing had indicated that oven-dried 2-wk-old plants were difficult to handle. Also, a highly positive correlation (r^2 = .998) was shown to exist between fresh and dry weights of seedlings in those tests.

M. hapla-infected seedlings weighed significantly less than did noninfested plants;

Table 1. Effects of nematode-bacterium interactions on fresh weights of 2-wk-old 'Saranac AR' alfalfa seedlings grown in interaction chambers.

Nematode	Number of nematodes per seedling	Mean fresh weights (mg.) of seedlings in the presence (+) or absence (–) of bacterium					
		*P. viridiflava**		*P. corrugata**		*P. marginalis**	
		–	+	–	+	–	+
M. hapla	0	46A†	43AB	44AB	40B	40B	40B
	12	39B	27DE	35C	31CD	35C	31CD
	25	34C	22E	38B	29D	38B	29D
	50	36BC	23E	33C	21E	33C	21E
P. penetrans	0	45A†	44A	39AB	41AB	41A	40AB
	12	43A	36B	38AB	35BC	38AB	37B
	25	40AB	32C	40AB	30C	37BC	34BC
	50	37B	29C	39AB	23D	34C	23D
H. dihystera	0	51A†	48AB	45AB	43CD	50A	46AB
	12	49A	47AB	40DE	42D	47AB	46AB
	25	45C	39DE	40DE	35EF	40CD	42CD
	50	41D	25G	38E	32F	38CD	41DE
D. dipsaci	0	50A	48A	52A	50A	46A	47A
	12	47A	45A	46A	46A	45A	44A
	25	40B	40B	38B	40B	36B	37B
	50	31C	33C	34C	31C	33C	33C

*Bacteria were applied as 1 ml of a suspension containing 2×10^8 cells per ml of single isolates.

†Values are means of 72 observations pooled from two experiments. Means (within the same nematode group) followed by the same letter are not significantly (P = 0.05) different according to Duncan's LSD test (k = 100).

bacterial infection alone did not cause significant weight reductions. Synergistic interactions between *M. hapla* and *P. viridiflava* and *P. corrugata* were indicated, especially in situations in which 25 or 50 nematodes per seedling were applied. Enhancement of *P. marginalis* damage by *M. hapla* was not observed.

Only when the plants were inoculated with 50 *P. penetrans* per seedling was there a significant reduction in weight compared to the controls. Synergistic interactions between the nematode and each of the test bacteria were indicated.

H. dihystera-infected plants inoculated with 25 and 50 nematodes per seedling caused a significant reduction in weight as compared to noninfected plants. Enhancement of weight reduction was observed in interactions with all of the test bacteria when 50 nemas per seedling were applied, but only with *P. syringae* and *P. corrugata* when 25 nemas per seedling were applied. This is the first report of pathogenicity to alfalfa of *H. dihystera*. The significance of this species to alfalfa culture in the field has not been investigated, but the results discussed here indicate that the potential exists for the production of significant damage to alfalfa by *H. dihystera*, alone or in disease complexes with soil microorganisms. No weight reductions due to bacteria alone were observed.

Seedlings in sand to which 25 or 50 *D. dipsaci* per seedling were applied weighed 16% and 33% less, respectively, than did noninfected plants. Bacteria alone did not result in weight reductions, and no interactions between *D. dipsaci* and any test bacteria were observed. The failure of *D. dipsaci* to interact with test bacteria may be related to its tendency to feed on foliar tissues, rather than on roots; preliminary tests indicated that the response of needle-inoculated green tissue of Saranac AR seedlings to the three test bacteria differs markedly from the response of roots or etiolated foliar tissue.

Preliminary experiments in which aseptically-grown Saranac AR seedlings were stab-inoculated with single bacterial isolates indicated that *P. viridiflava* caused the most severe root watersoaking and discoloration, and produced the greatest weight reductions of the three species tested; *P. marginalis* consistently produced the least severe injury and stunting. The results shown in Table 1 demonstrate that in interactions with the nematodes tested, the same order of virulence was observed.

Pot studies: The results of experiments conducted with older seedlings grown in greenhouse soil mix (Table 2) support conclusions drawn from experiments utilizing young seedlings in chambers. Single pathogens did not reduce the fresh weights of plant tops, compared with weights of noninfected plants in these studies. Among nematode-bacterium combinations, those including *P. viridiflava* and *P. corrugata* caused significantly greater reductions than those including *P. marginalis*. Significant reductions in top weight were observed for combinations involving all nematodes tested, except for *D. dipsaci*, as in chamber studies. The greatest weight reductions were

Table 2. Effects of nematode-bacterium interactions on fresh weights of 8-wk-old alfalfa seedlings in greenhouse soil mix.

Nematode*	Bacterium†	Mean fresh plant weight (g)‡
No nematodes	—	2.25 A
–	P.v.	2.09 A
–	P.c.	2.15 A
–	P.m.	2.19 A
M. hapla	—	2.03 A
"	P.v.	0.71 C
"	P.c.	0.95 C
"	P.m.	1.43 B
P. penetrans	—	1.98 A
"	P.v.	0.79 C
"	P.c.	0.80 C
"	P.m.	1.35 B
D. dipsaci	—	1.73 AB
"	P.v.	1.65 AB
"	P.c.	1.69 AB
"	P.m.	1.75 AB
H. dihystera	—	2.21 A
"	P.v.	1.30 B
"	P.c.	1.45 B
"	P.m.	2.10 A

*5,000 nematodes/pot.

†— = No bacteria used, P.C. = *P. corrugata*, P.v. = *P. viridiflava*, P.m. = *P. marginalis* (20×10^8 bacteria/pot).

‡Values are means of 40 observations, top weights only, pooled from two experiments. Means followed by the same letter are not significantly different according to Duncan's LSD test, with k = 100.

caused by combinations including *M. hapla* and *P. penetrans,* although significant plant weight reductions resulted from combinations including *H. dihystera.* These observations suggest that plant responses observed in interaction studies performed in chambers may be valid indicators of responses to be expected of older plants grown under less stringently controlled conditions.

Surveys of alfalfa field soil indicate that large numbers of nematode species are associated with the crop (6). Not all are detrimental to alfalfa, but may contribute to root disease by interacting with organisms of the root-rot complex. Recognized pathogenic nematode species may contribute to such complexes, in addition to exerting direct effects on the host. The results of these experiments suggest that such rhizosphere bacteria, including organisms not generally considered important pathogens of alfalfa, may play a significant role in the root-rot complex, in the presence of nematodes or other phytophagous soil organisms.

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