Infection of Seedlings of Alfalfa and Red Clover by Concomitant Populations of Meloidogyne incognita and Pratylenchus penetrans[1]

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Abstract: Invasion of 2-day-old seedlings of 'Buffalo' alfalfa and 'Kenland' red clover by larvae of *M. incognita* and adults of *P. penetrans*, during 1-3 day periods of incubation at 24 C, was investigated in 50-mm petri dishes on 1% agar. Penetration by both nematodes increased arithmetically with increased numbers in inocula. *P. penetrans* invaded alfalfa more readily than red cover, but *M. incognita* invaded red clover more readily than alfalfa. Both nematodes inhibited root-elongation of alfalfa more than that of red clover. In combinations of 10 and 50 of both nematodes, invasion of both plants by both nematodes was the same as for each nematode alone. Penetration by *M. incognita* into alfalfa, but not into red clover, was significantly reduced when combinations of 50 *M. incognita* and 200 *P. penetrans* were inoculated simultaneously. In the presence of large numbers of entrant *P. penetrans* in both plants, penetration by *M. incognita* was highly significantly reduced. Penetration by *P. penetrans* was unaffected in the reciprocal situations.

The root-knot nematode, *Meloidogyne incognita* (Kofoid and White), and the lesion nematode, *Pratylenchus penetrans* (Cobb), are both pathogenic endoparasites of many species of higher plants. However, their modes of parasitism are strikingly different. Second-stage larvae of *M. incognita* become established in roots in the region of differentiating vascular tissue after a brief period of migration through the cortex during which they incite little or no discernible damage to cells of the cortex (9, 12). At sites of establishment of larvae, cells in the stele develop abnormally into syncytia (22) that cause disruption of xylem strands and development of irregular xylem elements (9). Concurrently, as separate phenomena, hypertrophy and hyperplasia of cortical cells surrounding developing larvae result in the formation of galls. In contrast, *P. penetrans* usually invades roots behind the zones of cell elongation and differentiation (23) and remains in the cortex where it usually incites browning and necrosis of cells (26, 27, 34).

This study was designed to determine the extent of interaction between these two nematodes and to determine their effects on selected hosts during invasion and early parasitism. Red clover (*Trifolium pratense* L.) and alfalfa (*Medicago sativa* L.) were selected as hosts because they are both susceptible to *P. penetrans* (6, 7, 19), but differ in susceptibility to *M. incognita*. Red clover is more susceptible to infection by *M. incognita* than is alfalfa (2, 8, 28, 29).

MATERIALS AND METHODS

M. incognita was propagated on roots of tomato (*Lycopersicon esculentum* Mill. 'Rutgers') in a greenhouse. Egg masses were removed from 2- to 3-month-old plants and processed in two ways. They were placed in small beakers and barely covered with distilled water, or they were placed in a solution of 0.3

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M NaCl (4, 13). The former provided larvae for periods of 1-4 days. The latter, stored in NaCl for 4-15 days, were transferred to distilled water for 24 hr to stimulate hatching. Since root-knot nematode larvae lose infectivity rapidly when kept in water (3), only those hatched within a 24-hr period prior to inoculation were used.

P. penetrans was reared on alfalfa-seedling callus according to the method of Krusberg (21). Callus from 2- to 4-month-old cultures was placed in Baermann funnels and, in order to assure maximum infectivity, adults (males and females, 1:1) collected during periods not exceeding 24 hr were used for inocula (20, 31, 32). Most of the adults in a culture were collected during the first 4-6 days in the funnels, and males and females were obtained in approximately equal numbers.

Seeds of 'Kenland' red clover and 'Buffalo' alfalfa were surface-sterilized in 2.1% NaOCl for 10 min, rinsed in sterile tap water and transferred to sterile 90-mm petri dishes containing moist sterile discs of Miracloth® (Chicopee Mills, Inc., 1450 Broadway, New York, N.Y. 10018). The seeds were incubated for 48 hr at 20-25 C in the dark to obtain seedlings with primary roots 10-20 mm long. Single seedlings were placed in 50 mm (I.D. bottom) petri dishes containing approximately 4 ml of 1% agar.

Micropipettes were used to collect nematode inocula, and the suspensions of nematodes were placed on the agar at preselected sites. Excess water was allowed to evaporate and to be absorbed before the placing of seedlings on the agar. As the suspending fluid disappeared, the nematodes were drawn into a circle approximately 2-3 mm diam. Seedlings were placed in the petri dishes so that nematode inocula were 5-10 mm away from tips of roots in line with the direction of their growth.

Sterile glassware and agar and disinfested seedlings were used to reduce sources of contamination; however, the procedure ceased to be aseptic when the nematodes were added since they were not disinfested. Storing egg masses of *M. incognita* in NaCl reduced, but did not eliminate, contamination by various microorganisms. Liquid from nematode suspensions was added to all controls. Also, when inocula from different sources were used, appropriate treatments with nematode-free liquid were included. Preliminary testing had shown that the liquid in which nematodes were collected had no effect, when compared with water and no additive, on growth of the seedlings.

All seedlings were incubated in darkness at 24 C in B.O.D. incubators. This temperature is favorable for both *M. incognita* (5, 11) and *P. penetrans* (10, 24).

Prior to incubation, root lengths of seedlings were measured under a stereoscopic microscope. "Root" was arbitrarily designated to be that portion of a seedling between the root tip and the region where root hair formation ceased. The remainder of the plant was designated "hypocotyl" and "cotyledons".

After incubation, seedlings were prepared for microscopic examination according to a modification of McBryde's method (25). Seedlings were fixed individually in 10 ml 95% or absolute ethyl alcohol and glacial acetic acid (1:1, v/v) for 24 hr or longer. After fixing, each seedling was stained for 24 hr by adding 1 drop of 0.12% aqueous acid fuchsin to the fixative. They were cleared in concentrated aqueous chloral hydrate (4.5 g/ml) for about 2 hr prior to being mounted in clear lactophenol for microscopic examination.

Seedlings were examined by microscope fields at 100 X beginning at the root tip. The diameter of the field at 100 X was 1.5 mm; hence, each field represented a 1.5-mm segment of the seedling. Tissue discoloration, branch root initials, branch roots, galling, cell distortion and numbers of nematodes and their orientation were recorded by fields. Final root lengths were determined when the seedlings were examined microscopically.

Each treatment was replicated five times, and each experiment was repeated at least once unless noted otherwise. Student's t-test was used to determine significance of difference between means (30).

RESULTS

The relationships between numbers of nematodes in inocula and numbers that entered roots were arithmetic when each host was inoculated with each nematode alone. The line ($Y=a+bX$) that expresses the relationship for each combination was calculated from many coordinates obtained from 12, 9, 7 and 7 experiments with *M. incognita*-alfalfa, *M. incognita*-red clover, *P. penetrans*-alfalfa and *P. penetrans*-red clover combinations, respectively.

TABLE 1. Penetration of roots of alfalfa and red clover by larvae of *Meloidogyne incognita* and adults of *Pratylenchus penetrans* after 48 or 72 hr at 24 C.

Number in inoculum	Number in roots of Alfalfa Mi[a]	Alfalfa Pp[a]	Red clover Mi	Red clover Pp
6	3.7[b]	4.2	3.1	2.4
12	5.0	8.4	6.5	6.7
25	16.5	19.6	13.5	16.6
50	22.2	38.8	28.8	35.3
100	43.8	59.0	60.5	67.1
200	96.3	163.3	124.7	130.3

[a]Mi = M. incognita; Pp = P. penetrans.
[b]Data are means of five or more replicates.

The data in Table 1 are for selected numbers in inocula (values of X) within the ranges used. Correlation coefficients, r, and probabilities, *P*, were calculated to determine uniformity and significance of the data.

M. incognita. In the range of 4-200 larvae in inocula, the arithmetic relationships were very uniform and highly significant for *M. incognita* on both hosts. Values of r and *P* were 0.96 and <0.001 and 0.93 and <0.001 for alfalfa and red clover, respectively. Nearly maximal invasion of roots of both plants occurred in 48 hr, and extending the incubation period to 72 hr resulted in only a slight increase. Red clover (slope, b = 0.637) was invaded more readily than alfaifa (b = 0.472).

In seedlings of both plants, inoculated with low numbers (4-12/seedling) and incubated 48 or 72 hr, larvae appeared to have penetrated separately. Most of them occurred singly in individual 1.5-mm segments that frequently were widely separated throughout the plant. At higher inoculum levels, 20-200/seedling, penetration was *en masse* and the majority of larvae that invaded were in the first 3.0 mm of the root. At the two highest inoculum levels, 190 and 200/seedling, larvae of *M. incognita* frequently were packed (30-50/1.5-mm segment of root) among the arms of xylem in the stele for several millimeters behind the root tip. Farther up the root, fewer larvae were present, but they were still among the arms of xylem. Throughout the root, the larvae, with few exceptions, were oriented anteriorly away from the root tip. In the root-tip field, larvae frequently were observed entering the protostele. Thus, larvae of *M. incognita* appear to be capable of migrating relatively long distances through plants via the stele among the strands of mature xylem that are surrounded by cells lacking secondary-wall thickening.

During their apparent migration, larvae were confined to the stele and pericycle in the roots, but often they were in the cortex of the hypocotyl and appeared to be traversing cortical tissue. Occasionally they were observed invading the pericycle of the hypocotyl. As inoculum levels were increased, larvae were found in the hypocotyl with increasing frequency and in greater numbers per plant, and the uniformity of orientation was less pronounced than in root tissue.

At lower inoculum levels, slight enlargement of entrant larvae, an indication of their establishment and feeding, was observed frequently after 72 hr, but rarely after 48-hr incubation periods. The number of enlarged larvae found in red clover after 72 hr of incubation was two-three times greater than the number found in alfalfa in comparable treatments. At higher levels of inoculum, enlarged larvae rarely were found. Incubation periods of 48 and 72 hr were too brief for marked enlargement of established larvae. Root-galls were larger on red clover than on alfalfa.

P. penetrans. *P. penetrans* invaded roots of both hosts in greater numbers than did *M. incognita* (Table 1). Rates of penetration agreed with those reported previously (14, 17). The arithmetic relationships were very uniform and highly significant on both hosts. Values of r and *P* were .99 and <.001 and .96 and <.001 for alfalfa and red clover, respectively. The majority of *P. penetrans* invaded seedlings within 24 hr. Invasion continued between 24 and 48 hr, but there was little or no additional invasion after 48 hr. Alfalfa (slope, b = 0.811) was invaded more readily than red clover (b = 0.652).

In contrast to the lack of readily observable injury to root tissues incited by *M. incognita* during invasion, discoloration of epidermal and cortical tissues incited by invasion and feeding of *P. penetrans* was very pronounced. The location of *P. penetrans* in roots and the accompanying discolored tissues are accurate and valid measures of the sites of penetration because the areas traversed by the nematodes are no greater than the discolored areas (1). In no case did the areas of discoloration indicate that a nematode had moved more than 2 mm after entering.

When inocula contained from 3-50 adults/seedling, the principal sites of penetration by *P. penetrans* were within 4.5- 19 mm from the root tip. Nematodes were found to be concentrated in the first 12 mm when inoculum levels were increased to 81-200/seedling. The greater number of nematodes in the first 4.5 mm of roots of heavily inoculated plants, where they were not found in plants inoculated with lower numbers, suggests that they were migrating with the growing tip and/or were invading meristematic tissue of the root tip. There was no consistent relationship between sites of invasion and branch root initials.

M. incognita and P. penetrans. When alfalfa was inoculated simultaneously with combinations of 10 + 10, 10 + 50 and 50 + 50 of each species, penetration by both nematodes was unaffected. Data from six experiments, three incubated 48 hr and three incubated 72 hr, were consistent and are combined in Table 2. There were no significant differences among means within experiments. When red clover was inoculated simultaneously with combinations of 50 + 200 of each species, again penetration by both nematodes was unaffected, but when alfalfa was inoculated with the same combinations, penetration by *M. incognita* was significantly reduced in the combination containing 50 *M. incognita* plus 200 *P. penetrans*. Penetration by *P. penetrans* was unaffected in the reciprocal combination. Data from two experiments were consistent and are combined in Table 3.

TABLE 2. Penetration of roots of 48 hr-old seedlings of alfalfa by *Meloidogyne incognita* and *Pratylenchus penetrans* when inoculated simultaneously in various combinations. Seedlings were incubated 72 hr at 24 C.

Penetration by	In the presence of 0 Pp[a]	10 Pp	50 Pp
Mi[b]	4.2/10[c]	6.1/10	3.5/10
	19.7/50	26.6/50	18.9/50
	In the presence of 0 Mi	10 Mi	50 Mi
Pp	7.3/10	8.2/10	7.4/10
	38.8/50	41.3/50	43.2/50

[a]Pp = P. penetrans.
[b]Mi = M. incognita.
[c]Numerator = number of entrant nematodes; denominator = number of nematodes in inoculum. Data are means of three experiments.

To determine whether prior infection of alfalfa with large numbers of either species would affect invasion by the other, seedlings were inoculated singly with 200 *M. incognita* or 200 *P. penetrans* and incubated at 24 C for 48 hr and 24 hr, respectively. These incubation periods were chosen because most larvae of *M. incognita* invaded within 48 hr and most adults of *P. penetrans* invaded within 24 hr. After their respective incubation periods, seedlings were rinsed in sterile distilled water to remove nematodes that had not penetrated and then were transferred to petri dishes of fresh agar.

TABLE 3. Penetration of 48-hr-old seedlings of red clover and alfalfa by *Meloidogyne incognita* and *Pratylenchus penetrans* when inoculated simultaneously in various combinations of 50 and 200. Seedlings were incubated 48 hr at 24 C.

Penetration by	Into red clover in the presence of 0 Pp[a]	50 Pp	200 Pp	Into alfalfa in the presence of 0 Pp	50 Pp	200 Pp
Mi[b]	27.1/50[c]		16.9/50	25.7/50		8.4/50[d]
	129.3/200	117.4/200		96.1/200	75.5/200	
	Into red clover in the presence of 0 Mi	50 Mi	200 Mi	Into alfalfa in the presence of 0 Mi	50 Mi	200 Mi
Pp	33.9/50		38.7/50	37.4/50		40.3/50
	151.1/200	160.9/200		166.0/200	148.8/200	

[a]Pp = P. penetrans.
[b]Mi = M. incognita.
[c]Numerator = number of entrant nematodes; denominator = number of nematodes in inoculum. Data are means of 2 experiments.
[d]Difference significant at P = .05.

TABLE 4. Invasion of roots of 48-hr-old seedlings of red clover and alfalfa inoculated sequentially with reciprocal combinations of 50 and 200 *Meloidogyne incognita* and *Pratylenchus penetrans*. Seedlings were incubated at 24 C for 48 hr and 24 hr following inoculation with *M. incognita* and *P. penetrans*, respectively.

Penetration by	Into red clover previously invaded by		Into alfalfa previously invaded by	
	0 Pp[a]	93.8 Pp	0 Pp	145.0 Pp
Mi[b]	30.5/50[c]	11.7/50[d]	24.7/50	7.7/50[d]
	Into red clover previously invaded by		Into alfalfa previously invaded by	
	0 Mi	114.2 Mi	0 Mi	83.7 Mi
Pp	39.1/50	31.3/50	30.7/50	34.0/50

[a]Pp = P. penetrans.

[b]Mi = M. incognita.

[c]Numerator = number of entrant nematodes; denominator = number of nematodes in inoculum. Data are means of 2 experiments.

[d]Difference significant at P = .01.

Seedlings preincubated with 200 *M. incognita* were inoculated with 50 *P. penetrans* and incubated for an additional 24 hr. Those preincubated with 200 *P. penetrans* were inoculated with 50 *M. incognita* and incubated another 48 hr. There was a corresponding control treatment, nematode-free liquid from inoculum suspensions, for each inoculation treatment. Penetration by *M. incognita* into roots previously invaded by *P. penetrans* was significantly less than it was into comparable roots devoid of *P. penetrans* (Table 4). However, in the reciprocal treatment, *M. incognita* had no effect on the number of *P. penetrans* that invaded.

TABLE 5. Effects of *Meloidogyne incognita* and *Pratylenchus penetrans* on elongation of roots of alfalfa and red clover after 48 or 72 hr at 24 C.

No. entrant nematodes	Elongation (mm) of roots of uninoculated minus elongation of roots of inoculated			
	Alfalfa		Red clover	
	Mi[a]	Pp[a]	Mi	Pp
1-19.9	4.9[b]	4.1	1.7	2.6
20-39.9	7.4	6.4	3.8	2.8
40-59.9	10.6	9.4	0.2	4.3
60-79.9	10.7	10.0	7.2	2.4
80-99.9	5.6		8.8	10.2
100-119.9	13.9		6.3	5.6
120-139.9	18.3	18.3	5.6	11.8
140-159.9		20.3		7.3
160-179.9		14.5		

[a]Mi = M. incognita; Pp = P. penetrans.

[b]Data are means of five or more replicates.

This experiment was repeated with red clover seedlings. Results correlated closely with those with alfalfa. Invasion by *M. incognita* was significantly lowered in the presence of large numbers of previously entrant *P. penetrans* (Table 4). The reciprocal inoculation sequence did not inhibit invasion by *P. penetrans*.

Differences between elongation of uninoculated minus elongation of inoculated roots were used as indications of gross effects of the nematodes on the two hosts. The relationships between numbers of entrant nematodes and reduction of elongation were arithmetic when each host was inoculated with each nematode alone. The lines that express the relationships were calculated as for the penetration data. The data in Table 5 are for selected groups of numbers of entrant nematodes (values of X) within the ranges used. These data are not as consistent as those for penetration. Most of this variability is accounted for by inherent variation in root elongation among seedlings. Coefficients of variability for uninoculated controls in three experiments were virtually the same within experiments as between experiments. Values of r and *P* were .54 and .05-.01 and .22 and >.05 for *M. incognita* and *P. penetrans*, respectively, on red clover. Values of r and *P* were .56 and <.001 and .84 and <.001 for *M. incognita* and *P. penetrans*, respectively, on alfalfa. Elongation of roots of red clover was reduced by similar amounts by both nematodes (slope, b = 0.041 and 0.048 for *M. incognita* and *P. penetrans*, respectively). Likewise, elongation of roots of alfalfa was reduced by similar

amounts by both nematodes (b = 0.104 and 0.100 for *M. incognita* and *P. penetrans*, respectively). Reduction of root elongation of alfalfa was approximately twice that of red clover (b = .100-.104 vs. .041-.048).

DISCUSSION

M. incognita penetrated roots of red clover more rapidly and abundantly than roots of alfalfa; the amount of development of entrant larvae and root galling were greater in red clover than in alfalfa; and inhibition of root elongation was less in red clover than in alfalfa. These results confirm that red clover is a more suitable host than alfalfa for *M. incognita*. *P. penetrans* invaded roots of alfalfa more abundantly than roots of red clover, and inhibition of root elongation was greater in alfalfa than in red clover. Data for penetration of both nematodes into both hosts were very consistent, but data for inhibition of root elongation were variable, especially for red clover. Despite this difficulty, we believe these data are useful for indicating the relative effects of these nematodes on these hosts. The effects are consistent with the results from experiments in which concomitant populations of the nematodes were used.

Penetration of *M. incognita* into alfalfa, the less suitable and more sensitive host for this nematode, was significantly reduced when 50 *M. incognita* were inoculated simultaneously with 200 *P. penetrans*. The same combination on red clover, the more suitable and less sensitive host for *M. incognita*, produced a consistent, but not significant, reduction in penetration by *M. incognita*. Penetration by *M. incognita* into both red clover and alfalfa when their roots contained substantial numbers of previously entrant *P. penetrans* was highly significantly reduced. *P. penetrans*, which invades more rapidly and in equal (red clover) or greater (alfalfa) numbers than *M. incognita*, made the epidermis and cortex of roots unsuitable for larvae of *M. incognita*. The degree of antagonism increased as *P. penetrans* was given an advantage in numbers and time.

This effect was not evident when combinations of lower numbers, 10 and 50, were inoculated simultaneously onto both hosts. At these inoculum levels, *P. penetrans* did not congregate in the region of cell elongation. Consequently, this region, the site most suitable for invasion by *M. incognita*, was not as severely injured as it was when large numbers of *P. penetrans* invaded and many of them settled in this region.

Penetration by *P. penetrans* was not affected by disproportionately large numbers of concomitant or previously entrant *M. incognita*. The effects of invading larvae on cells of the cortex (15) were not deleterious to invading *P. penetrans*.

Estores and Chen (16) reported that population development of *P. penetrans* in tomato was repressed in the presence of *M. incognita*. In their work, plants were maintained in pots in a greenhouse, and experiments had a duration of 36 and 60 days. *P. penetrans*, an amphimictic species, has a generation time of 54-65 days and produces about 16-35 progeny/female (18, 24). Females lay eggs at the rate of 1-2/day (18, 24, 36). In contrast, species of *Meloidogyne* are parthenogenetic. The generation time of root-knot nematodes is 20-30 days (10, 33), and a female is capable of producing 500 eggs at the rate of 27-120/day (35). Therefore, in mixed infestations, the population of *M. incognita* would be expected to increase more rapidly than that of *P. penetrans*, but repression of population development of *P. penetrans* by *M. incognita* would not be expected within 36-60 days. Some factor that reduced the reproductive rate of *P. penetrans* in mixed infections below that of infections of *P. penetrans* alone had to be operating.

Our results and the results of Estores and Chen (16) appear to be contradictory. However, the differences between conditions, hosts, laboratory vs. greenhouse and short vs. long periods of time of the two sets of experiments are too great to permit comparison. The physiological and morphological changes that *M. incognita*-infected roots undergo as the nematode matures may have significant effects on the development of *P. penetrans*. Longer-term experiments are now underway to determine what these effects are.

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