Infection of Red Clover Seedlings by Heterodera trifolii Goffart and Pratylenchus penetrans (Cobb)[1]

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Abstract: Penetration of 3-day-old 'Kenland' red clover seedlings by *Heterodera trifolii* Goffart and *Pratylenchus penetrans* (Cobb) was investigated in 50-mm petri dishes on 1% agar or discs of Miracloth® (Chicopee Mills, Inc., New York, N.Y. 10018). Penetration by both nematodes increased arithmetically with increased numbers in the inoculum. *H. trifolii* larvae slowly penetrated all root tissue but in relatively low numbers, 25-30% of inocula. Swelling of larvae and formation of syncytia within roots was detectable 96 hr after inoculation. Initially, adults of *P. penetrans* preferentially penetrated the region 3-10 mm behind the root tip, but subsequently they invaded all along roots. *P. penetrans* penetration efficiency was high, 75-90% of inocula, and penetration was relatively rapid. When introduced simultaneously neither nematode affected the invasiveness of the other. Prior infection by *H. trifolii* did not affect the invasiveness of *P. penetrans*. *Key Words:* Concomitant infection.

The root lesion nematode, *Pratylenchus penetrans* (Cobb), and the clover cyst nematode, *Heterodera trifolii* Goffart, are pathogens (1, 2, 7, 9) of red clover (*Trifolium pratense* L.) and their concomitant association with this host in Kentucky is frequent. *P. penetrans* is a migratory endoparasite that incites necrosis of tissue in which it moves and feeds (8, 14) whereas *H. trifolii* is a sedentary endoparasite that incites the development of syncytia (3, 6, 10). However, according to Mankau and Linford (12), syncytia develop slowly, and larvae moved about as long as 2 weeks before settling down. This means that both species are migratory endoparasites during the initial stages of development within roots.

The purpose of this study was to determine the relative rates of penetration and early development of these nematodes, both alone and in combination, in roots of red clover.

MATERIALS AND METHODS

P. penetrans and *H. trifolii* were obtained from colonies that had been maintained on 'Atlantic' and 'Buffalo' alfalfa (*Medicago sativa* L.) and 'Kenland' red clover, respectively, for several years in a greenhouse. Roots of stock plants, not more than 4 months old, were hung in a mist chamber (1) and only those nematodes collected during the 24-hr period prior to inoculation were used. Active nematodes for inocula were collected by allowing them to flow into capillary pipettes.

Inocula consisted of adults of *P. penetrans* and second-stage larvae of *H. trifolii*. Adults of *P. penetrans* (1:1 females:males) were used because they are more invasive than larvae (16) and approximately equal numbers of females and males occurred in collections from the stock plants.

Clover seed (cv. 'Kenland') were immersed in 2.1% NaOCl for 5 min, rinsed in sterile water, and placed on sterile 90-mm discs of moistened Miracloth® (Chicopee Mills, Inc., 1450 Broadway, New York, N.Y. 10018) in sterile petri dishes. Miracloth is a coarse non-woven fabric of cellulose fibers. Seeds were germinated and incubated in the dark for 72 hr at 20-25 C. This procedure provided seedlings with roots 1-2 cm long. Single seedlings were placed between two saturated (ca. 1.2 ml water) Miracloth discs or on 1% agar in 50-mm (I.D. bottom) petri dishes.

Two procedures were used to inoculate seedlings on Miracloth. Random inoculations were made by adding the nematodes to the water which was used to saturate the discs. Localized inoculations were made by placing small droplets of suspensions at desired sites on previously saturated discs. Localized inoculations were used on agar. Droplets of suspensions were placed at desired sites and excess water was absorbed by the agar. Seedlings then were placed with root tips close to but not within the sites of inoculum deposition. The droplets used in the localized inoculations spread over a circular area ca. 1 cm in diameter on both media. Although sterilized glassware and water and disinfested seedlings

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were used in order to reduce contamination, the procedure was not aseptic because the nematodes were not disinfested.

Inoculated seedlings were incubated in the dark for 72 hr at 20-25 C unless specified otherwise.

A modification of the method of McBryde (11) was used to prepare seedlings for microscopic examination. After incubation, seedlings were fixed and stained 24 hr in 10 ml 1:1 (*v/v*) absolute ethyl alcohol and glacial acetic acid containing one drop of 0.12% aqueous acid fuchsin, cleared in concentrated aqueous chloral hydrate (3.6 g/ml) for ca. 2 hr, and then mounted in clear lactophenol.

Seedlings were examined at 100 X. Numbers and locations of nematodes, branch root initials, branch roots and areas of discoloration and cell distortion were recorded by fields starting at the root tip. The diameter of the microscope field was 1.1 mm and, therefore, observations were made on each 1.1-mm segment of seedlings. The region from the tip of the root cap to the end of the piliferous zone was designated "root," and the region beyond the root to the cotyledons was designated "hypocotyl."

All experiments contained five replicates unless stated otherwise and were repeated at least once. Significance of differences between means was determined by Student's t-test (5).

RESULTS

Greater number of *H. trifolii* invaded seedlings on Miracloth from localized inoculations than from random inoculations (14-43% of inocula in 12 experiments vs. 5% of inocula in two experiments). *P. penetrans* also was more invasive on Miracloth from localized inoculations than it was from random inoculations (70-96% of inocula in 10 experiments vs. 59-60% in two experiments). In addition, significantly (t=0.01) greater numbers of *H. trifolii* invaded seedlings from agar than from Miracloth (mean of 52% of inocula in 14 experiments vs. 22% of inocula in 12 experiments). There was no significant difference in the invasiveness of *P. penetrans* from the two media (mean of 91% of inocula on Miracloth vs. 84% of inocula on agar in two experiments each). Incubation was 72 hr for *H. trifolii* and 24-72 hr for *P. penetrans* at 20-25 C.

H. TRIFOLII ALONE: Larvae of *H. trifolii* invaded red clover slowly. As incubation time was increased from 24 to 72 hr, the number of larvae that invaded roots usually increased. A few attempts showed that during incubation periods longer than 72 hr (96 and 120 hr) no significant additional increase was obtained.

There was significant (P=0.01) arithmetic increase (y=0.813 + 0.229 X) in the number of larvae in roots of red clover as the number in the inoculum was increased from 2-108 per plant. This equation for the relationship was calculated from the combined data from five experiments containing five replicates each. The relationship within each experiment was significant (P=0.01).

Based on discoloration and/or distortion of cells that result from activity of the nematodes in roots, movement within roots was confined within 2-mm segments during incubation periods. Since the larvae had moved no more than 2 mm from their positions at the time of observation, their locations at this time were considered to be reasonable measures of sites of penetration.

At low inoculum levels, three and nine nematodes per plant, larvae penetrated roots independently of each other within a distance of 5-16 mm from the root tip (Fig. 1-A, B). However, as inoculum levels were increased to 27 and 81 nematodes per plant, larvae were found closer to the root tip and in the hypocotyl (Fig. 1-C, D). In contrast to penetration at low inoculum levels, where the mean number of larvae per 1.1-mm segment of root was less than one, penetration at high inoculum levels showed clustering near the root tip where the mean numbers of larvae per 1.1-mm segment of root ranged from less than one to three. The sites of penetration at all inoculum levels were not associated with sites of branch roots or branch root initials.

Swelling of larvae in the roots, which is evidence of their feeding, was not noticeable prior to 72 hr after penetration. After 96 hr, swelling of some larvae was noticeable, and after 120 hr most of the larvae showed evidence of having fed. Syncytia appeared at the same time swelling of larvae occurred.

P. PENETRANS ALONE: Adults of *P. penetrans* penetrated roots more rapidly than larvae of *H. trifolii*. As the incubation period was increased to 72 hr, the number of nematodes that penetrated increased, but the amount of increase after 48 hr was not great.

There was a significant (P=0.01) arithmetic increase (y=0.164 + 0.760 X) in the number

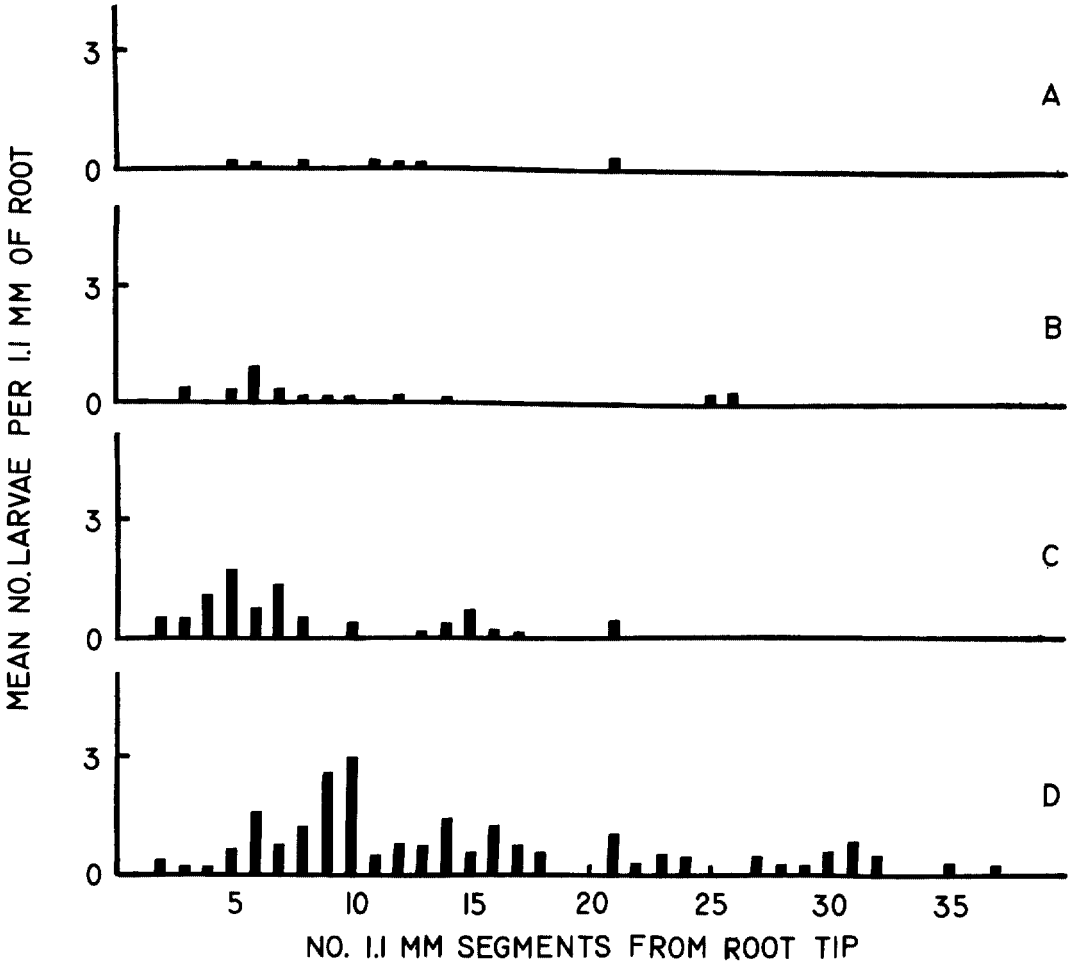

FIG. 1. Mean numbers of *H. trifolii* per 1.1-mm segment in red clover seedlings inoculated at root tips. Seedlings were incubated with 3 (A), 9 (B), 27 (C) and 81 (D) larvae on agar for 72 hr at 20-25 C. The mean numbers of larvae that penetrated A, B, C and D were 1.4, 3.4, 9.6, and 21.0, respectively. The maximum mean number of larvae in any one segment was 3.0. Data are means of five replicates.

that penetrated as the number in the inoculum was increased over the range of 2-40 nematodes per plant. The equation for the relationship was calculated from the combined data of four experiments containing five replicates each. The relationship within each experiment was significant (P=0.01). Preliminary experiments with inoculum levels of 50 and 100 adults indicated that this relationship holds up to the level of 100 nematodes per seedling. El-Sherif and Mai (4) obtained similar results with *P. penetrans* and alfalfa.

The same criteria as those used for *H. trifolii* were used to determine sites of invasion by *P. penetrans*. Discoloration was more evident in roots invaded by *P. penetrans* than in those invaded by *H. trifolii*. Based on these criteria, the total length of root through which individual nematodes moved during incubation was ca. 2 mm from their position at the time of observation. However, cell damage within the limits of this area was more extensive than it was in roots invaded by *H. trifolii*, indicating that *P. penetrans* moved about within the limits of the area more extensively than did *H. trifolii*. Since the nematodes had moved no more than 2 mm from their position at the time of observation, their locations at this time were considered to be reasonable measures of sites of penetration.

The principal sites of penetration by *P. penetrans* were within 3-11 mm of the root tip during a 24 hr incubation period when 50 nematodes were placed near the root tip (Fig. 2-A). During a longer incubation period (72 hr) nematodes continued to congregate in the same

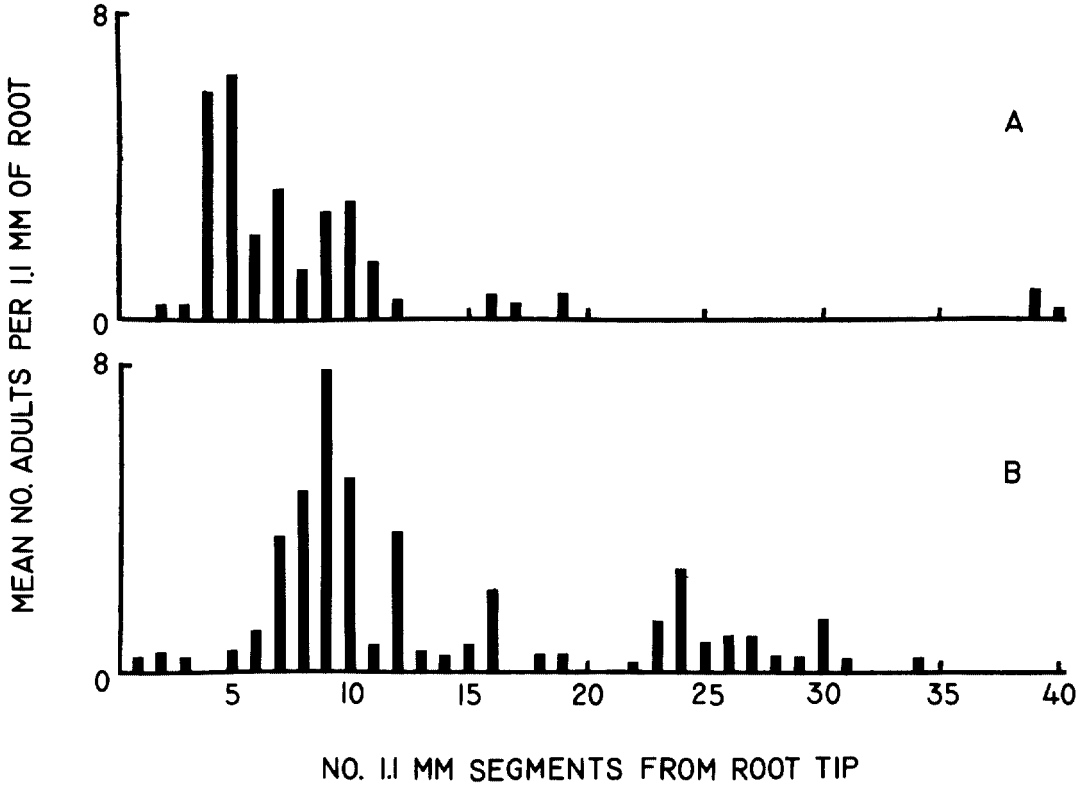

FIG. 2. Mean numbers of *P. penetrans* per 1.1-mm segment in red clover seedlings inoculated with 50 adults at root tips. Inoculated seedlings were incubated for 24 hr (A) and 72 hr (B) at 20-25 C. The mean numbers of adults that penetrated A and B were 30.4 and 39.8, respectively. The maximum mean number of larvae in any one segment was 7.8. The mean root-hypocotyl junctures were located in segments 21 and 27 of A and B, respectively. Data are means of five replicates.

area (6-13 mm) that was occupied during 24 hr, and, in addition, they penetrated all along the root toward the root-hypocotyl juncture and occasionally (1% in 24 hr vs. 6% in 72 hr) into the hypocotyl (Fig. 2-B). They did not penetrate readily the newly developed root tissue distal to the previously invaded site and there was no consistent relationship between sites of penetration and sites of branch roots or branch root initials.

H. TRIFOLII AND *P. PENETRANS:* Equal numbers of *H. trifolii* and *P. penetrans* (2, 5, 10, 20 each) were used in localized inoculations at tips of roots of seedlings in five experiments on Miracloth. Corresponding numbers of each species alone were included as controls. Each control contained liquid from the inoculum of the other species so that fungal and bacterial contamination introduced by both species was effected when each species was used singly. This liquid was collected in capillary pipettes from nematode suspensions. Nematodes were not allowed to flow into the pipettes. Rates of penetration and distribution within roots were the same for each species alone and in combination. Under these conditions, neither species significantly affected penetration of the other. There was no tendency for individuals of the different species to be closely associated with or isolated from each other. Possibly, *H. trifolii* was at a disadvantage in these experiments because of its low penetration rate on Miracloth. However, the results were confirmed by experiments in which varying numbers of *P. penetrans* (5, 10, 20, 40) were used with constant numbers of *H. trifolii* (5 or 20) in localized inoculations on agar (Table 1).

In the preceding experiments, the plants were inoculated with the two species simultaneously. Miller and Wihrheim (13) found that, in tobacco (*Nicotiana tabacum* L.), *Heterodera tabacum* Lownsbery and

TABLE 1. Penetration by *P. penetrans* and *H. trifolii*, in various combinations, into red clover seedlings incubated on agar for 72 hr at 20-25 C. In the fractions, numerators denote numbers penetrated and denominators denote numbers in inocula. Data are means of three replicates.

In the presence of *H. trifolii* (no.)	Penetration by *P. penetrans* was			
0	3.3/5	7.0/10	13.0/20	23.0/40
5	2.7/5	6.0/10	15.0/20	29.0/40
0	2.0/5	7.0/10	10.6/20	31.8/40
20	3.0/5	7.8/10	15.6/20	24.2/40

In the presence of *P. penetrans* (no.)	Penetration by *H. trifolii* was	
0	4.2/5	5.0/20
5	2.7/5	8.6/20
10	2.5/5	10.2/20
20	2.7/5	10.1/20
40	4.2/5	7.2/20

Lownsbery repressed population development of *P. penetrans* during a period of 4 weeks. In order to determine whether prior invasion by *H. trifolii* affected subsequent invasion by *P. penetrans* in red clover, 30 larvae of *H. trifolii* were inoculated near the root tips of seedlings on agar and incubated for 72, 96 or 120 hr. Then 20 adults of *P. penetrans* were placed near the root tips and the seedlings were incubated for an additional 48 hr. Controls consisted of seedlings inoculated with equivalent amounts of liquid (no nematodes) in which the larvae of *H. trifolii* had been suspended. Penetration by *P. penetrans* was the same whether or not the seedlings had been invaded previously by *H. trifolii* (Table 2), and the distribution of *P. penetrans* within the seedling was unaffected by the presence of *H. trifolii*.

TABLE 2. Penetration of roots of red clover, previously incubated with larvae of *H. trifolii* (H) for 72, 96 and 120 hr, by adults of *P. penetrans* (P) on agar at 20-25 C. Incubation was for an additional 48 hr following introduction of *P. penetrans*.

Incubation period (hr)	Inoculum (no.)		Penetration (no.)	
	H	P	H	P
72[a]	0	20		11.2
	20	0	10.6	
	20	20	7.4	11.9
96[b]	0	20		8.8
	20	0	8.8	
	20	20	9.7	11.8
120[c]	0	20		10.8
	20	0	7.3	
	20	20	6.2	8.8

[a] Means of three experiments containing five replicates each.
[b] Means of two experiments containing five replicates each.
[c] Means of nine replicates in two experiments.

DISCUSSION

The early stages of penetration and development by *H. trifolii* in clover roots was previously described by Mankau and Linford (12). They found red clover to be a poor host for *H. trifolii* and observed prolonged wandering by larvae before establishment in roots. In contrast to their results, we observed development of syncytia and evidence of feeding shortly after invasion, and long periods of wandering did not occur. These differences cannot be reconciled at this time. They used a population of *H. trifolii* that was not well adapted to red clover, whereas we used one that was well adapted to this host (15). Also, they inoculated 20-day-old plants growing in sand, whereas we used 3-day-old plants in an *in vitro* system.

Neither nematode affected the invasiveness of the other when they were inoculated simultaneously or when inoculation of *H. trifolii* was 72-120 hr prior to that of *P. penetrans*. If population development of *P. penetrans* is repressed by *H. trifolii* in red clover as it was by *H. tabacum* in tobacco after four weeks (13) it is not because *H. trifolii* reduces root penetration by *P. penetrans*. In later development it is possible that physiological changes stimulated by developing females of *H. trifolii* within host tissue may be such that continued development of *P. penetrans* would be inhibited.

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