

## Differential Response of Thor Alfalfa to *Meloidogyne chitwoodi* Races and *M. hapla*<sup>1</sup>

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**Abstract:** Second-stage juveniles (J2) of races 1 and 2 of *Meloidogyne chitwoodi* and *M. hapla* readily penetrated roots of Thor alfalfa and Columbian tomato seedlings; however, few individuals of *M. chitwoodi* race 1 were able to establish feeding sites and mature on alfalfa. Histopathological studies indicate that J2 of race 1 either failed to initiate feeding sites or they caused cell enlargement without typical cell wall thickening. The protoplasm of these cells coagulated, and juveniles of race 1 did not develop beyond the swollen J2 stage. A few females of race 1 fed on small giant cells and deposited a few eggs at least 20 and 30 days later than *M. chitwoodi* race 2 and *M. hapla*, respectively. Failure of race 1 to establish feeding sites was related to egression of J2 from the roots. The *M. chitwoodi* race 1 J2 egression from alfalfa roots was higher than egression of race 2 and *M. hapla*. Egression of J2 of *M. chitwoodi* races 1 and 2 from tomato roots was similar and higher than that of *M. hapla*. Thus egression plays an important role in the host-parasite relationship of *M. chitwoodi* and alfalfa.

**Key words:** alfalfa, Columbia root-knot nematode, egression, histopathology, *Meloidogyne chitwoodi*, *M. hapla*, northern root-knot nematode, physiological race, resistance.

The Columbia root-knot nematode (*Meloidogyne chitwoodi* Golden et al. 1980) is an important parasite of potato (15). Extensive host-range studies indicate that alfalfa (*Medicago sativa* L.) is not a suitable host to the type culture of *M. chitwoodi* (11). Thus, alfalfa is recommended as a rotational crop with potato to reduce field populations of *M. chitwoodi* (15). An isolate of this nematode capable of reproducing on alfalfa roots, however, was recently reported as the alfalfa race (race 2) of *M. chitwoodi* (16). The alfalfa race has been found in all of the major potato growing regions of the Pacific Northwest (12).

Studies on the reproductive efficiency of different *M. chitwoodi* isolates on alfalfa displayed a continuum from nonhost to suitable host (12). One report indicated that nematodes of both races could damage selected alfalfa cultivars (6). These results suggest that both races are capable of invading alfalfa roots, but race 1 either fails to induce giant cells and dies or leaves the alfalfa roots after penetration. Egression of *M. incognita acrita* (Kofoid and White)

Chitwood (13) and *M. hapla* Chitwood (5) from resistant alfalfa roots has been reported. The nature of active resistance of plants to root-knot nematode infection has been reviewed (3,7).

Objectives of the studies reported here were to examine the rate of penetration of, and egression from, roots of alfalfa cultivar Thor by second-stage juveniles (J2) of *M. chitwoodi* race 1 and 2, and to observe the histological changes in alfalfa roots in response to penetration by J2 of *M. chitwoodi* races. *M. hapla*, a known parasite of alfalfa, was included for comparison.

### MATERIALS AND METHODS

The experiments were conducted with *Meloidogyne* isolates from the Irrigated Agriculture Research and Extension Center collection (12). The behavior of *M. chitwoodi* race 1 (WAMc1), race 2 (ORMc8), and *M. hapla* were compared on alfalfa, *Medicago sativa* cv. Thor.

The nematodes were reared on tomato, *Lycopersicon esculentum* Mill cv. Columbian. Nematode eggs were obtained from tomato roots by the NaOCl method (8). J2 were collected and stored according to Vrain (17).

Alfalfa and tomato seeds were germinated at 24 C for 48 hours before they were planted in methyl bromide-fumigated sandy loam soil (84% sand, 10% silt, 6%

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clay) or in 250- $\mu$ m-d (60-mesh) washed river sand. The treatments were arranged in randomized blocks or split plots with 5–20 replicates. The data were transformed to log or arcsin scale when necessary (9), and an analysis of variance and F-tests were performed to determine the differences.

*Reproductive efficiency:* Three-week-old alfalfa (three per pot) or single tomato seedlings were transplanted into 10-cm-d clay pots filled with sandy loam soil, and 500 J2 of *M. chitwoodi* race 1, or race 2, or *M. hapla* suspended in 5 ml water were added to each pot. The pots were maintained at 24 C with 14-hour photoperiod for 55 days. Reproduction efficiency was assessed by washing the roots free of soil, staining with Phloxin B (4), and counting egg masses. Eggs were extracted (8) and quantified.

*Penetration and egression:* Germinated alfalfa and tomato seeds were planted in 100-compartment seed-soaking trays (Neogen Food Tech, Lansing, MI) containing 5 cm<sup>3</sup> river sand per compartment. Each compartment received 1 ml water at planting. Trays were transferred to boxes covered with clear plastic bags to minimize moisture loss and stored in a 24 C incubator with 14-hour photoperiod for 1 week before nematode inoculation. Seedlings were irrigated once with three or four drops of water. Fifty seedlings of either plant species were inoculated with each nematode isolate. Each seedling received 200 J2 suspended in 1 ml water. They were incubated for 4 days after inoculation. The sand was soaked with water, seedlings were gently removed, and the roots were washed free of sand. Fifteen alfalfa and tomato seedlings per nematode isolate were immediately stained, and the number of J2 penetrating alfalfa or tomato roots was determined. Another set of 15 alfalfa and tomato seedlings was transplanted into 7.5-cm-d plastic cups containing sandy loam soil. After 3 weeks in the greenhouse, the roots were washed free of soil and stained with acid fuchsin and the number of nematodes was determined. The percentage of J2 that egressed was calculated based on

the numbers that remained after 3 weeks relative to the number that penetrated the first set of plants. A third set of 15 alfalfa and tomato seedlings was incubated in tap water for 72 hours, and the J2 egressing from the roots were collected daily and counted. They were then placed in soil around tomato roots to ascertain their infectivity.

*Postinfection nematode development:* In one experiment, alfalfa seedling roots were exposed to 200 *M. chitwoodi* or *M. hapla* J2 in seed soaking trays for 4 days. The seedlings were transplanted to 7.5-cm-d cups containing sandy loam soil and maintained at 24 C in temperature controlled water tanks for 3 weeks. The cups were removed from the tanks, and the phenology of invading nematodes was studied by staining the invaded root with acid fuchsin (2). Five nematode life stages were quantified: vermiform J2, swollen J2, pegged third-stage and fourth-stage juveniles, young female (immediately after the fourth molt), and mature female (pear shaped). In another experiment, alfalfa seedlings were exposed to 800 J2 of each nematode species at 24 C for 60 days. Every 5 days two cups per nematode species were harvested, and the first appearance of nematode life stages in stained roots was recorded. Invaded root segments were excised and fixed in formaldehyde-acetic-alcohol (10 parts 38% formaldehyde, 2 parts acetic acid, and 20 parts 95% ethyl alcohol in 100 parts distilled water). They were dehydrated in ethyl alcohol and embedded in parawax, sectioned at 15  $\mu$ m with a rotary microtome, and stained with safranin and fast green according to Berlyn and Miksche (1), except that Hysto-Clear (National Diagnostic, Somerville, NJ) was substituted for xylene. The sections were mounted in a synthetic mounting medium and examined with a compound microscope.

## RESULTS

*Reproductive efficiency:* *M. chitwoodi* race 1 reproduced efficiently on tomato but produced very few egg masses and eggs on alfalfa (Table 1). Conversely, *M. chitwoodi*

TABLE 1. Egg masses and eggs (numbers) produced by *Meloidogyne chitwoodi* races 1 and 2 and *M. hapla* on Thor alfalfa and Columbian tomato 55 days after inoculation with 500 second-stage juveniles.

	Alfalfa		Tomato	
	Egg masses	Eggs ( $\times 10^3$ )	Egg masses	Eggs ( $\times 10^3$ )
<i>M. chitwoodi</i>				
Race 1	1.2 a	0.071 a	141.0 a	89.0 a
Race 2	31.1 b	15 b	140.0 a	81.4 a
<i>M. hapla</i>	101.7 b	73 b	317.2 b	356.6 b

Values are means of five replicates. Values in each column followed by the same letter are not significantly different ( $P = 0.05$ ) according to Duncan's multiple range test.

race 2 and *M. hapla* reproduced well on both hosts. *M. hapla* produced more ( $P < 0.05$ ) egg masses and eggs on tomato than did the *M. chitwoodi* races (Table 1). Alfalfa roots infected with *M. chitwoodi* were swollen but seldom galled, and root branching was rare. *M. hapla* caused extensive galling and branching of alfalfa roots, and multiple infections were more common than with *M. chitwoodi*.

*Penetration and egression:* Although tomato had more ( $P < 0.05$ ) lateral roots per plant than did alfalfa (eight vs. two), the number of J2 penetrating each root system was similar, ranging from 20 to 70 in 4 days. Prolonging the exposure of plants to nematodes did not increase the rate of penetration. No differences ( $P > 0.05$ ) in the ability to penetrate alfalfa or tomato roots were observed between *M. chitwoodi* races and *M. hapla* (Tables 2, 3).

When invaded roots were incubated in water for 72 hours, a large percentage of all three groups of nematodes egressed from tomato roots (Table 2). Significantly ( $P < 0.05$ ) more J2 of *M. chitwoodi* race 1 egressed from alfalfa roots, relative to *M. hapla*. Egression of *M. chitwoodi* race 2 did not differ from either race 1 or *M. hapla* (Table 2). Percentage of J2 of *M. chitwoodi* race 1 egressing from alfalfa roots 3 weeks after invaded seedlings were planted in soil was higher ( $P < 0.05$ ) than percentage of race 2 or *M. hapla* J2 (Table 3). The egression of *M. chitwoodi* races 1 and 2 from tomato roots was higher ( $P < 0.05$ ) than *M.*

TABLE 2. Second-stage juveniles of *Meloidogyne chitwoodi* races 1 and 2 and *M. hapla* penetrating Thor alfalfa or Columbian tomato roots after 4 days and egressing after incubation of roots in water for 4 days.

	Alfalfa		Tomato	
	Pene- trated† (N)	Egressed‡ (%)	Pene- trated† (N)	Egressed‡ (%)
<i>M. chitwoodi</i>				
Race 1	27 a	86.7 a	33 a	99.0 a
Race 2	17 a	79.7 ab	22 a	79.3 a
<i>M. hapla</i>	18 a	54.6 b	56 a	99.2 a

Values are means of 10 replicates. Values in each column followed by the same letter are not significantly different ( $P = 0.05$ ) according to Duncan's multiple range test.

† Inoculum density = 200 J2/plant.

‡ Number of J2 left the roots  $\div$  number penetrated  $\times 100$ .

*hapla* egression. Bioassay on tomato showed that 25, 33, and 57% of the J2 of *M. chitwoodi* races 1 and 2 and *M. hapla*, respectively, which egressed from alfalfa were able to penetrate and establish feeding sites in tomato roots.

*Postinfection nematode development:* J2 of *M. chitwoodi* races and *M. hapla* invaded alfalfa root tips and caused swelling at infection sites. These root-tip swellings became galls with extensive root proliferation by *M. hapla* but not by *M. chitwoodi*. Initially (4 days), multiple infection at root tips was common for all nematodes; however, later observations showed that *M. chitwoodi* were commonly located singly along the root axis

TABLE 3. Second-stage juveniles of *Meloidogyne chitwoodi* race 1 and 2 penetrating Thor alfalfa or Columbian tomato roots within 4 days and egressing from roots 3 weeks after transplanting into soil.

	Alfalfa		Tomato	
	Pene- trated† (N)	Egressed‡ (%)	Pene- trated† (N)	Egressed‡ (%)
<i>M. chitwoodi</i>				
Race 1	50 a	85.3 a	27 a	74.0 a
Race 2	68 a	39.0 b	53 a	62.1 a
<i>M. hapla</i>	51 a	12.4 b	59 a	27.0 b

Values are means of 11 replicates. Values in each column followed by the same letter are not significantly different ( $P = 0.05$ ) according to Duncan's multiple range test.

† Inoculum density = 200 J2/plant.

‡ Number of J2 penetrated  $\div$  number of nematodes remaining  $\div$  number of J2 penetrated  $\times 100$ .

TABLE 4. Thor alfalfa root system in which different life stages of *Meloidogyne chitwoodi* races and *M. hapla* were found 3 weeks after inoculation with 200 J2.

	Plants infected (%)	Nemas examined (N)	Different stages (%)				
			J2	Swollen J2	J3-J4	Young ♀	Mature ♀
<i>M. chitwoodi</i>							
Race 1	30	14	43	36	21	0	0
Race 2	60	67	7	3	3	47	40
<i>M. hapla</i>							
	60	66	0	0	0	8	92

Twenty plants per population examined.

and *M. hapla* remained in groups within galls. Discolored root tissue surrounding females of both *M. chitwoodi* races was evident after staining with acid fuchsin. No evidence of root discoloration was observed with *M. hapla*.

Most of the *M. chitwoodi* race 1 J2 in alfalfa roots were surrounded by tissue deeply stained with safranin (Fig. 1A) and failed to establish a feeding site. Some J2, however, initiated feeding sites and began to enlarge. Most of these feeding sites soon became atypical, the cell walls failed to thicken, and the protoplasm coagulated (Fig. 1B). Nematodes associated with these atypical cells did not develop beyond the swollen J2. Few individuals of *M. chitwoodi* race 1 were able to maintain the integrity of their feeding sites. The mean  $\pm$  standard error of these giant cells were smaller ( $691 \pm 131 \mu\text{m}^2$ ) than those initiated by *M. chitwoodi* race 2 ( $2,545 \pm 377 \mu\text{m}^2$ ) or *M. hapla* ( $5,638 \pm 470 \mu\text{m}^2$ ) (Fig. 1C, E, F). *M. chitwoodi* race 1 females that fed on these small giant cells developed slowly and produced few eggs (Fig. 1D).

After 3 weeks more alfalfa root systems contained *M. chitwoodi* race 2 and *M. hapla*—and these nematodes were in more advanced growth stages—than *M. chitwoodi* race 1 (Table 4). Only 30% of the roots contained race 1, and 43% of the nematodes were still vermiform J2; none were beyond J4. In contrast, over 60% of the roots contained *M. chitwoodi* race 2 or *M. hapla*, a majority of which were either young or adult females. A larger percentage (92%) of these *M. hapla* developed to maturity than *M. chitwoodi* race 2 (40%).

The swollen J2 and J3-J4 stages of all three groups of nematodes were first observed 10 and 15 days after inoculation, respectively (Table 5). Adult females of *M. hapla* and *M. chitwoodi* race 2 and race 1 were observed 18, 25, and 30 days after inoculation, respectively. The eggs deposited by these mature females were detected 2, 5, and 20 days later (Table 5).

#### DISCUSSION

The higher level of reproduction of *M. chitwoodi* race 2 on Thor alfalfa, relative to race 1, substantiates our earlier report (12) on the differential host preference of these two races. The differential behavior of *M. chitwoodi* races on alfalfa appears regulated by several processes. The failure of *M. chitwoodi* race 1 to establish on alfalfa was caused partially by egression of the nematodes soon after penetration. The method used to determine egression, however, may affect the number of J2 that egress. Reynolds et al. (13) suspended alfalfa roots in water and concluded that the failure of *M.*

TABLE 5. Days until the different developmental stages of *M. chitwoodi* races or *M. hapla* were observed in alfalfa roots inoculated with 800 second-stage juveniles (J2) and maintained at 24 C.

	Swollen			Mature female	Eggs laid
	J2	J2	J3-J4		
<i>M. chitwoodi</i>					
Race 1	5	10	15	30	50
Race 2	5	10	15	25	30
<i>M. hapla</i>					
	5	10	15	15-20	20

Invaded roots were harvested at 5-day intervals, stained, and examined with compound microscope.

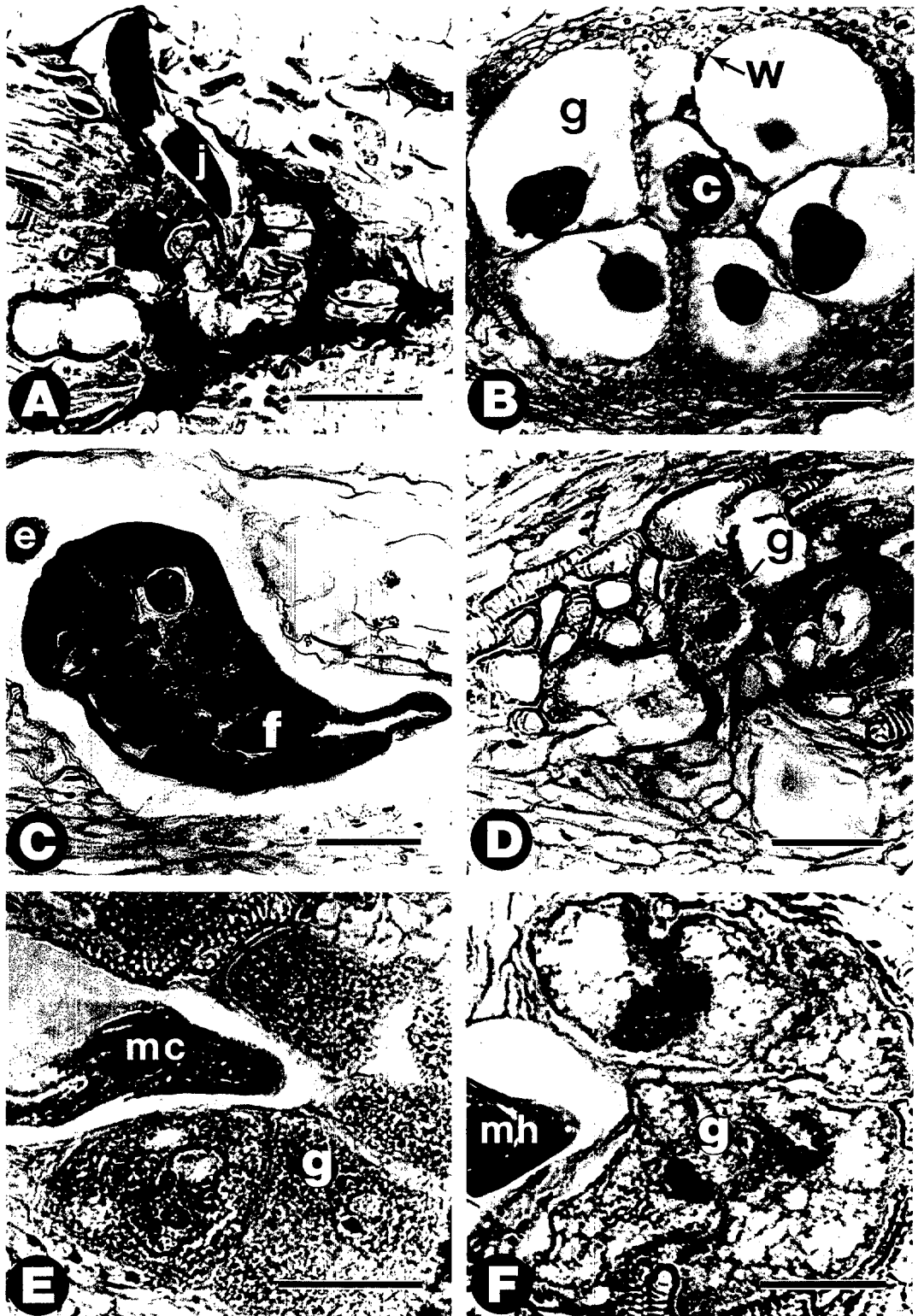


FIG. 1. Alfalfa infected with *Meloidogyne chitwoodi* race 1 (MC1) and 2 (MC2) and *M. hapla* (MH). A) MC1 J2 (j) failed to form giant cells. Note darkened tissue stained with safranin. B) Abnormal giant cells (g) produced

*incognita acrita* to establish on resistant alfalfa cultivars was caused by egression of J2 from the resistant roots. McClure et al. (10) used the same technique and observed that *M. incognita* J2 egressed from susceptible as well as resistant cotton roots. They rejected the idea that egression is responsible for failure of *M. incognita* to establish on resistant cotton. When invaded roots were suspended in water, *M. chitwoodi* and *M. hapla* J2 egressed en masse from the suitable tomato roots. A similar technique is used to extract migratory endoparasitic nematodes from roots. When seedlings exposed to the nematodes were transplanted to clean soil, J2 egressed mainly from the unsuitable host.

Histological studies showed that most *M. chitwoodi* race 1 J2 that did not leave alfalfa roots were immobilized in the vascular cylinder and surrounded by tissue darkly stained with safranin, an indication of changes in the histochemistry of the host and possible necrosis (14). Tissue discoloration was common in alfalfa roots invaded by both races of *M. chitwoodi* but not in roots invaded by *M. hapla*.

A few *M. chitwoodi* race 1 J2 initiated small giant cells in alfalfa roots and produced a few eggs. Thus, continuum reproduction of different populations of *M. chitwoodi* on Thor alfalfa reported earlier (12) appears to be related to the number of these J2 that establish feeding sites and (or) the number of eggs they eventually deposit. The host status of Thor alfalfa to the type culture of *M. chitwoodi* (WAMc1) that was used here, however, remained unchanged, and it failed to establish on Thor alfalfa even after three consecutive sequential inoculations (unpubl.).

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by MC1. Note nonthickened cell walls (w) and coagulated cytoplasm (c). C) Undersized MC1 female (f) with a single egg (e) 50 days after inoculation. D) Single, undersized, functional giant cell (g) produced by MC1. E, F) MC2 (mc) and MH (mh) females feeding on normal giant cells (g). All bars = 50  $\mu$ m.

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