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Characterization of Root-Knot Nematode Resistance in *Medicago truncatula*

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Abstract: Root knot (*Meloidogyne* spp.) and cyst (*Heterodera* and *Globodera* spp.) nematodes infect all important crop species, and the annual economic loss due to these pathogens exceeds $90 billion. We screened the worldwide accession collection with the root-knot nematodes *Meloidogyne incognita*, *M. arenaria* and *M. hapla*, soybean cyst nematode (SCN-*Heterodera glycines*), sugar beet cyst nematode (SBCN-*Heterodera schachtii*) and clover cyst nematode (CLCN-*Heterodera trifolii*), revealing resistant and susceptible accessions. In the over 100 accessions evaluated, we observed a range of responses to the root-knot nematode species, and a non-host response was observed for SCN and SBCN infection. However, variation was observed with respect to infection by CLCN. While many cultivars including Jemalong A17 were resistant to *H. trifolii*, cultivar Paraggio was highly susceptible. Identification of *M. truncatula* as a host for root-knot nematodes and *H. trifolii* and the differential host response to both RKN and CLCN provide the opportunity to genetically and molecularly characterize genes involved in plant-nematode interaction. Accession DZA045, obtained from an Algerian population, was resistant to all three root-knot nematode species and was used for further studies. The mechanism of resistance in DZA045 appears different from *Mi*-mediated root-knot nematode resistance in tomato. Temporal analysis of nematode infection showed that there is no difference in nematode penetration between the resistant and susceptible accessions, and no hypersensitive response was observed in the resistant accession even several days after infection. However, less than 5% of the nematode population completed the life cycle as females in the resistant accession. The remainder emigrated from the roots, developed as males, or died inside the roots as undeveloped larvae. Genetic analyses carried out by crossing DZA045 with a susceptible French accession, F83005, suggest that one gene controls resistance in DZA045.

Key words: root-knot nematode, cyst nematode, resistance, *Medicago truncatula*, host range, genetics.

It has been estimated that the average yield loss due to parasitic nematodes is around 12% annually (Sasser and Freckman, 1987), reaching as high as 20% in certain crops (Koenning et al., 1999). Among the parasitic nematodes, root-knot nematodes (RKN) and cyst nematodes are the most important and wide spread. Every crop species grown is susceptible to one or more RKN species (Sasser, 1980). RKN (*Meloidogyne* spp.) are obligate sedentary endo-parasites and are known to occur across a broad range of climatic conditions. While *Meloidogyne* contains more than 70 described species, four species (*M. incognita, M. arenaria, M. javanica* and *M. hapla*) are responsible for 95% of infestations (Sasser et al., 1983). The genus *Heterodera* includes several economically important nematodes. For instance, the yield loss due to SCN (*Heterodera glycines*) in soybean is estimated to be $430 million in US alone for the year 1995 (Wrather et al., 1997). Similarly, the annual economic loss due to sugar beet cyst nematode (SBCN-*Heterodera schachtii*) is estimated to be $95 million in Europe alone (Muller, 1999). While no clear estimates are available for yield losses caused by *H. trifolii*, it has been reported that it can infect a number of crops, including sugar beet and other vegetable crops (Wang and Riggs, 1999), and also suppresses nitrogen fixation in many leguminous plants (Yeats et al., 1977).

RKN initiates a feeding site after the infective second-stage larva (L2) has penetrated the host root, generally near the root tip, and migrated to the developing vascular cylinder. The nematode induces formation of five to seven giant cells within or near the developing vascular cylinder, which become the permanent feeding site (Hussey, 1989). The nematode is dependent upon the giant cells for its survival and reproduction because it becomes immobile soon after giant cell induction. RKN gets its common name from the classic symptom of heavy root galling in the areas of infection. These external symptoms are pronounced and diagnostic.

Infective second-stage larvae of cyst nematodes penetrate young roots directly and move intra-cellularly to the cortex, where they initiate specialized feeding sites. The cyst nematode feeding site is called a synctium and is formed by the degradation of cell walls and cell hypertrophy (Hussey, 1989). Once the nematode establishes a syncytium, it undergoes three molts and become an adult. During the development of the third-stage larvae, cortical cells surrounding the female larvae are crushed by its expanding body, exposing the nematode to soil. Although *H. schachtii* and *H. glycines* reproduce by amphimixis, *H. trifolii* reproduces by apomixis (Triantaphyllou and Hirschmann, 1978).

Compared to recent advances in other plant-pathogen interactions, less is known about the interaction between the nematode and its host. Although *Arabidopsis thaliana* (Sijmons et al., 1991; Boiteux et al., 1999; Vercauteren et al., 2001; Gheysen and Fenoll, 2002) and *Lotus japonicus* (Lohar and Bird, 2003; Lohar et al., 2004) have been used to probe susceptible inter-

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actions with RKN, virtually no work has been performed on RKN or cyst resistance in these plants, largely because no *Arabidopsis* or *Lotus* ecotypes have been demonstrated to be fully resistant to either nematode (Niebel et al., 1994). Consequently, we explored using *Medicago truncatula* as a model to understand resistant interactions between legume hosts and root-knot/cyst nematodes. *Medicago truncatula* is a diploid legume and a close relative of alfalfa. It has a small, completely sequenced genome (450Mb) and has proven to be an effective model to study the interactions between legumes and rhizobial bacteria (Barker et al., 1990; Dhandaydham, 2000; Frugoli and Harris, 2001, www.Medicago.org). Apart from its suitability as a model host, *M. truncatula* is widely grown as a pasture legume in Australia, although the geographical centre of origin for *M. truncatula* is the Mediterranean basin and the Near East (Crawford et al., 1989). Numerous accessions have been cataloged from wild isolates, and substantial genetic variability revealed (Auricht et al., 1999). Here we report the establishment of *M. truncatula* as a model to study the resistant interactions between legumes and nematodes.

Materials and Methods

Biological material: Medicago truncatula seeds were scarified in concentrated H_2SO_4 for 6 min, washed for 3 hr with several changes of sterile tap water and incubated at 4°C for 24 hr to synchronize germination. Seeds were washed again in sterile water for 3 hr and left in a germination chamber for 2 d at 26–28°C. Newly emerged seedlings were planted in 606 Com-Packs (Hummert International, Earth City, MO) that contained a pasteurized 4:1 mixture of sand and soil, and plants were maintained in the greenhouse or growth chamber. Three to four weeks after planting, plants were inoculated with nematodes by making four 1-inch-deep holes around the plant and placing approximately 250 nematode eggs in each hole. Six weeks after inoculation, plants were harvested, washed and evaluated using a galling index (Hussey and Janssen, 2002). For each accession, two to six plants were inoculated.

Nematode culture maintenance: H. glycines OP50 was maintained on soybean cv. Lee 74, *H. schachtii* was maintained on beet root cv. Monohikari, and *H. trifolii* 12JI (single J2 descendent generated from pine tree, Arkansas, provided by Dr. R.D. Riggs) was maintained on white clover. Nematode inoculum from the susceptible host plants was isolated as follows: first, the plant roots were soaked in water for few seconds to remove the soil from the root ball. Cleaned roots were later sprayed with high-pressure water over a sieve arrangement of 25/60 mesh. In addition, the cysts in the soil were collected by stirring the soil in water and decanting over the same sieve arrangement. Cysts collected over the 60 mesh sieve were crushed using a rubber stopper over a sieve arrangement of 60/200/500 mesh. The eggs collected over the 500 mesh sieve were counted and adjusted to a concentration of 1,500 eggs/ml for *H. trifolii*, 4,000 eggs/ml for *H. schachtii* and 8,000 eggs/ml for *H. glycines*.

Populations of *M. incognita* race 1, *M. arenaria* race 1 and *M. hapla* race A were maintained in greenhouse on *Lycopersicon esculentum* cv. Rutgers Large Red. Nematodes were extracted from roots with 0.5% NaOCl (Hussey and Barker, 1973). Eggs collected from a 500 mesh sieve were washed and resuspended at 10,000 eggs/ml.

Evaluation of response to root-knot nematodes: RKN egg masses were stained by placing galled roots in phloxine B solution (0.15 g/liter tap water) for 15–20 min. After staining, excess stain was removed by washing the roots in tap water, and the egg masses were counted using a stereo microscope (Daykin and Hussey, 1985). For rapid RKN screening, each plant was rated for galling and assigned a root galling index value with 0 = no galling, 1 = trace infections with a few small galls, 2 = <25% roots galled, 3 = 25–50% galling, 4 = 50–75% galling, and 5 = > 75% of roots galled. We consider a score of 2 or more to indicate susceptibility and one of less than 1 to indicate resistance. Evaluation of response to cyst nematodes was similar to the evaluation criteria set by Noel et al. (1990) enumerating cysts produced, and the accessions were grouped into resistant (0–15 cysts), mildly susceptible (16–50 cysts) and susceptible (> 50 cysts).

Penetration and emigration experiments for root-knot nematodes on resistant accessions: One-week-old *M. truncatula* seedlings grown on Pro-Trays containing 2:1 sand and soil mixture were inoculated with 500, 1,000 or 2,000 hatched L2. To determine the number of nematodes that had entered into the plant, five to eight seedlings of DZA045 (resistant) and F83005 (susceptible) were removed at 24, 48 and 96 hr after inoculation and stained with acid fuchsin. Acid fuchsin stains both nematodes and dead plant cells, including those resulting from an HR.

For emigration experiments, plants were harvested 1 wk after inoculation, washed free of soil and debris and maintained in a hydroponic culture in small cups containing 100 ppm of nitrogen:phosphorous:potassium (NPK 20:20:20). Once every 2 d, the hydroponic solution was filtered using a 10-μm-pore sieve, and the nematodes collected were counted using a stereo microscope. Larvae that left the root were collected from the hydroponic solution and counted every 2 d for 6 wk, although L2 were no longer observed outside the roots by 3 wk after inoculation. Nematode development was monitored during staining of root systems and the developmental stage noted.

Genetics: Recombinant inbred lines (RIL) are single-seed descendents of a cross between two homozygous parents that have been selfed for at least five genera-

tions (F6). RIL are nearly homozygous and homogenous and provide large numbers of individuals in which the parental alleles are expected to be distributed equally (Burr and Burr, 1991). We initially screened 42 F6 RIL with *M. incognita* race 1. Two sets of six plants were inoculated with 1,000 and 2,000 eggs, respectively, and analyzed for a visible resistance phenotype 6 wk later (Fig. 1). The reactions of the RIL were scored based on a galling index (0–5 scale). If the average galling score of an inbred line was ≤ 1, then that line was classified as resistant, and if the score was ≥ 3, then the inbred line was classified as susceptible. Lines that had an average score of 1.1 to 2.9 were classified as intermediate.

Because the galling index is to a degree subjective, we compared its reliability with that of egg mass production (which is a quantitative measure of relative nematode fecundity) by counting the number of egg masses present on the roots of the same plants by staining them with phloxine B. For resistant phenotypes, the galling index measure was highly reliable and comparable with that of egg mass numbers, so we used the galling index for subsequent experiments, as it is easier and faster to perform. To ensure the reliability of plant gall index phenotypes, we allowed the nematodes to complete two life cycles in the host. However, only 93.75% of the lines would be expected to have reached homozygosity in the F6 generation. To test the amount of heterozygosity in these RIL, we determined the segregation ratio for the phenotypic character *pod coiling* in these lines.

Results

Medicago truncatula is non-host for H. glycines and H. schachtii: A need for new and novel sources of resistance to SCN in soybean, the availability of numerous accessions of model legume, *M. truncatula* and the difficulty in carrying out genetic studies with tetraploid soybean prompted us to test whether *M. truncatula* can be used as a model to understand SCN-host interactions. In our experiments, we infected the plants with SCN inbred line OP50 at 1,000 eggs/plant. OP50 carries a large suite of necessary parasitism genes for infection of a variety of soybean cultivars (Dong and Opperman, 1997). Even 6 wk post-inoculation, no developing cysts were observed in any of the plants tested. We tested the accessions again with a larger inoculum (8,000 eggs/plant) during summer to verify this result. While developing nematodes were observed in the control soybean plants, no cysts were observed in any of the *M. truncatula* plants. A subset of these accessions was tested again by Dr. Terry Niblack, University of Illinois, Urbana, with SCN inbred line TN2, which has a host range which includes tomato. While most of the accessions were non-host to this SCN inbred as well, a few nematodes did develop on the accessions E39, E103 and F20025–2. However, the nematodes that grew on these lines could not be increased despite maintaining them for several months. Since *M. truncatula* was not a host for SCN, we tested the same accessions by infecting with SBCN at an inoculum of 4,000 eggs/plant. Similar to SCN, no SBCN cysts grew in any of the *M. truncatula* plants. In contrast, nematodes did develop on the susceptible cabbage plants, suggesting that *M. truncatula* was not a host for SBCN.

Medicago truncatula is a host for H. trifolii: Six weeks after infecting the plants with 1,500 CLCN eggs, the roots were checked for the presence of developing nematodes. Out of the 74 *M. truncatula* accessions tested, 16 supported growth of CLCN, the remaining 58 (~80%) were resistant (Table 1), indicating that *M. truncatula* is a host for CLCN (Table 1). However, out of the 11 *M. truncatula* commercial cultivars (distinct from accessions) tested, only four were resistant to the nematode. In fact, the cultivar Paraggio was the most susceptible (> 500 cysts) among all the cultivars and accessions together tested. Accessions C009, D231, E163, F11013 and F20009 showed high variability for CLCN infection, confirming that these accessions were indeed mixtures. Out of the three *Medicago littoralis* accessions tested, all the accessions were resistant to CLCN (Table 1).

Medicago truncatula accessions are genetically diverse and responded differentially to distinct RKN species: We initially inoculated *M. truncatula* with *M. incognita* race 1, reveal-

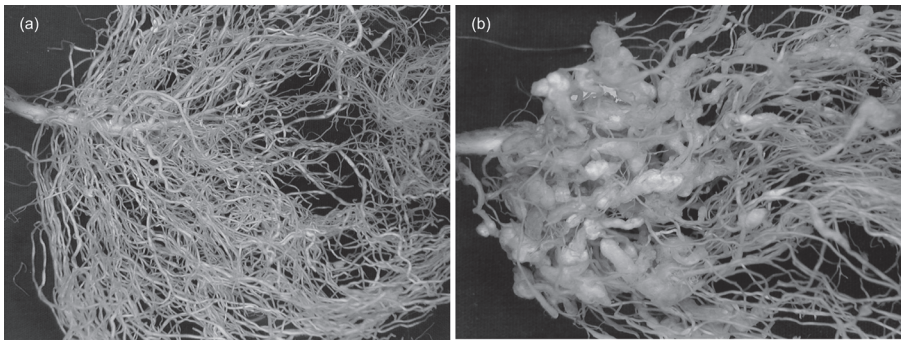

Fig. 1. Whole roots of *Medicago* accessions 12 weeks after inoculation with *M. incognita*. DZA045, the resistant plant (A) shows no symptoms, whereas root knots (galls) are clearly evident on a susceptible plant (F83005, gall score = 5) (B).

TABLE 1. *M. truncatula* accessions and their reaction to clover cyst nematode (*H. trifolii*) infection.

Accession	Origin	Number of cysts	S/R
C005	Crete	1	R
C006	Crete	0	R
C007	Crete	>200	S
C009	Crete	10	MS
D012	Algeria	0	R
D022	Algeria	2	R
D027	Algeria	0	R
D033	Algeria	2	R
D045	Algeria	0	R
D046	Algeria	0	R
D055	Algeria	8	R
D058	Algeria	0	R
D059	Algeria	0	R
D105	Algeria	0	R
D212	Algeria	0	R
D215	Algeria	0	R
D220	Algeria	0	R
D230	Algeria	0	R
D231	Algeria	12	R
D233	Algeria	0	R
D241	Algeria	0	R
D242	Algeria	0	R
D244	Algeria	28	MS
D246	Algeria	40	MS
D248	Algeria	1	R
D309	Algeria	0	R
D312	Algeria	2	R
D323	Algeria	0	R
E045	Spain	0	R
E048	Spain	0	R
E050	Spain	0	R
E074	Spain	0	R
E080	Spain	46	MS
E095	Spain	3	R
E096	Spain	0	R
E098A	Spain	0	R
E098B	Spain	0	R
E099A	Spain	1	R
E100	Spain	0	R
E101	Spain	0	R
E103	Spain	1	R
E104	Spain	0	R
E105	Spain	0	R
E140	Spain	3	R
E155	Spain	4	R
E156	Spain	0	R
E159	Spain	>75	S
E160	Spain	0	R
E161	Spain	>100	S
E162	Spain	0	R
E163	Spain	40	MS
E165	Spain	0	R
E169	Spain	18	MS
E170	Spain	0	R
E171	Spain	0	R
E172	Spain	0	R
E173	Spain	0	R
F11013	France	28	MS
F20009	France	32	MS
F20015	France	>200	S
F20031	France	3	R
F20047	France	45	MS
F20087	France	4	R
F83005	France	20	MS
G042	Greece	0	R
G043	Greece	0	R
G052	Greece	>100	S
G065	Greece	22	S
G093	Greece	0	R
G098	Greece	0	R
P176	Portugal	0	R
P177	Portugal	0	R
P179	Portugal	0	R
P180	Portugal	0	R
M. littoralis			
E032	Spain	1	R
E094	Spain	0	R
G036	Greece	0	R
Cultivars			
JEMALONG		0	R
A17		0	R
BORUNG		28	MS
CALIPH		35	MS
CYPRUS		50–100	S
HARBINGER		25	MS
MOGUL		18	MS
PARABINGA		0	R
PARAGGIO		>500	S
SEPHI		0	R
AMPUS		21	MS

0–15 = Resistant; 16–50 = Mildly susceptible; >51 = Susceptible

ing 50 of the 80 accessions scored to be resistant, 21 lines to be highly susceptible and the remaining nine accessions to be moderately susceptible to this RKN isolate. As shown in Figure 1, resistant and susceptible accessions are clearly discernable. The spectrum of genetic diversity between accessions we observed is shown in Table 2. To ensure that the differential resistance was not a consequence of growth conditions or RKN inoculum size, subsets of these accessions (40) were independently re-infected with 1,000 or 4,000 *M. incognita* race 1 eggs in a different greenhouse; the results (data not shown) were consistent with the previous findings. Although individual accessions were collected from single sites, they correspond to a sample of seeds collected from several individuals (Bonnin et al., 1996). Thus, because *M. truncatula* is an autogamous species, it is expected that each accession is a mixture of several individual inbred lines. Nevertheless, we found that many of the accessions were uniformly resistant to *M. incognita* race 1, suggesting that these accessions are under constant nematode pressure to retain resistance. This was not unexpected, since a previous survey (Taylor et al., 1982) had demonstrated that RKN is a common root pathogen in the Mediterranean basin. However, we observed substantial variation within each accession to infection and susceptibility to powdery mildew (data not shown), suggesting heterogeneity for traits not under continuous selection.

TABLE 2. *M. truncatula* accessions and their responses to RKN ifection. Lines with prefix DZA are from Algeria, CRE are from Crete, ESP are from Spain, GRC are from Greece, and P are from Portugal. Gall Index Scroes: S = Susceptible (>2.0), MS = Mildly Susceptible (1.1–1.9), R = Resistant (<1), ND = Not Determined.

Accession	*M.i.*[a]	*M.a.*[b]	Accession	*M.i.*[a]	*M.a.*[b]	Accession	*M.i.*[a]	*M.a.*[b]
A17	R	R	DZA246	ND	S	ESP165	ND	S
A20	R	ND	DZA248	ND	R	ESP169	ND	R
BORUNG	R	S	DZA309	ND	S	ESP170	ND	R
CALIPH[c]	S	R	DZA312	ND	R	ESP171	ND	R
CYPRUS	MS	R	DZA323	ND	R	ESP172	ND	R
HARBINGER	R	R	DZA327	ND	R	ESP173	ND	R
MOGUL	R	S	ESP031	R	R	ESP174	ND	S
PARABINGA	R	S	ESP039	R	ND	ESP175	ND	S
PARAGGIO	R	R	ESP040	ND	S	F11005	S	S
SEPHI	MS	S	ESP041	ND	R	F11007	MS	ND
AMPUS	S	ND	ESP043[d]	R	R	F11008	R	R
CRE005	R	R	ESP045	R	R	F11012	S	ND
CRE006[d]	S	S	ESP048	S	R	F11013	R	R
CRE007	R	ND	ESP050	S	R	F20009	S	ND
CRE009	R	R	ESP074	ND	R	F20025	R	ND
DZA012	S	R	ESP080	R	R	F20031	MS	ND
DZA016	R	R	F34042[c]	S	S	F20047	S	S
DZA022	S	R	F83005[c]	S	S	F20048	S	R
DZA027	R	R	GRC020	R	ND	F20058	R	R
DZA033	ND	R	GRC033	R	S	F20061	R	S
DZA045[d]	R	R	ESP095	R	R	F20069	ND	R
DZA046	S	S	ESP096	S	R	F20081	R	R
DZA055	R	R	ESP098A	S	S	F20086	MS	R
DZA058	MS	R	ESP098B	ND	S	F20087	MS	R
DZA059	R	R	ESP099A	R	S	F20089	S	S
DZA061	ND	R	ESP100	R	S	GRC037	R	R
DZA202	R	R	ESP101	ND	S	GRC040	MS	S
DZA210	R	S	ESP103	R	ND	GRC043	R	R
DZA213	R	R	ESP104	R	R	GRC052	ND	S
DZA219	R	R	ESP105	ND	R	GRC063	S	S
DZA222	ND	R	ESP140	ND	S	GRC064	R	S
DZA230	R	R	ESP155	R	R	GRC065	S	R
DZA231	ND	R	ESP156	R	S	GRC093	MS	S
DZA233	ND	S	ESP158	S	ND	GRC098	R	S
DZA236	R	R	ESP159	R	ND	PRT176	R	S
DZA241	R	R	ESP160	ND	R	PRT177	R	R
DZA242	ND	S	ESP161	ND	R	PRT178	ND	R
DZA243	ND	S	ESP162	ND	R	PRT179	ND	R
DZA244	ND	S	ESP163	ND	R	PRT180	R	S

[a] *M. incognita* inoculated with 1,000 or 2,000 eggs;
[b] *M. arenaria* with 4,000 eggs;
[c] Susceptible to *M. hapla* inoculated with 2,500 eggs.
[d] Resistant to *M. hapla* inoculated with 2,500 eggs.

Because the tomato *Mi* gene confers resistance to three different nematode species (Williamson, 1998), we further tested the *M. truncatula* accessions with *M. arenaria* and *M. hapla* (Table 2). As was the case for *M. incognita*, resistant and susceptible accessions were found for both species, but, strikingly, resistance to one RKN species was not predictive of resistance to another (Table 2); *Medicago* accessions show different combinations of resistance and susceptibility to different *Meloidogyne* species.

One accession (DZA045) collected from Algeria was broadly resistant to all three RKN species tested, and one French accession (F83005) was broadly susceptible. These were chosen for further analysis using *M. incognita*. Because the accessions retain some heterogeneity, we selected individual DZA045 (DZA045–5) and F83005 (F83005–5) plants and selfed them for two generations. Homozygosity of these progeny was confirmed by simple sequence repeat analysis (data not shown). Individual plants were inoculated with 1,000 *M. incognita* L2, and gall indices obtained 6 wk later (sufficient for one nematode life cycle). To further quantify the resistance/susceptibility of these accessions, we determined the reproduction factor, which is the ratio between the final nematode counts (Pf) to that of initial inoculum (Pi). For F83005–5, Pf/Pi = 20, confirming the high RKN-susceptibility of this accession indicated by the gall index. In contrast, we obtained Pf/Pi = 0.8 for the resistant accession, DZA045–5. Collectively, these data confirm that the plants that were chosen accurately represented the response of the accession.

Resistant accession DZA045 does not mount a Hypersensi-

tive Response: The hypersensitive response (HR) is a rapid response that resistant plants commonly mount against invading pathogens and usually results in programmed cell death of the plant cell at the initial point of infection. In tomato, the *Mi* gene conditions resistance to RKN infection and within 24 hr kills the cell where the nematode has initiated its feeding site (Dropkin, 1969). To determine whether the resistance in DZA045 is due to a HR, we inoculated 3-wk-old plants with 1,000 *M. incognita* L2 and stained the roots with acid fuchsin 24, 48 and 67 hr later; nematodes and dead plant cells acquire color from the stain, but live plant cells do not (Daykin and Hussey, 1985; Isghougi Kaloshian, personal communication). We observed multiple independent plants for evidence of a HR, but none showed any obvious symptoms (Fig. 2). It has been shown in tobacco that a HR can occur even after giant cell formation (Powell, 1962), so we stained the inoculated plants at various time points for up to 3 wk after inoculation, but at no point was a HR observed (not shown).

RKN penetrate susceptible and resistant accessions equally: Because resistance in DZA045 did not appear to correspond to a HR-based resistance mechanism, we tested whether it was due to lack of nematode penetration into the roots, which has been found to be the basis for RKN-resistance in cucumber (Haynes and Jones, 1976). As is the case on other hosts, RKN penetrate *Medicago* just behind the root tip. No meaningful difference in nematode penetration between resistant and susceptible accessions was observed (Fig. 3).

More nematodes emigrate from the resistant accession than from the susceptible accession: In grape root-stocks, resistance to RKN is positively correlated with the exit of nematodes from the root (Anwar and McKenry, 2002). Although approximately twice as many L2 emigrated from the resistant accession compared to the susceptible accession (Table 3), most nematodes remained in the roots (data not shown). Consequently, larval emigration does not explain the resistance in DZA045.

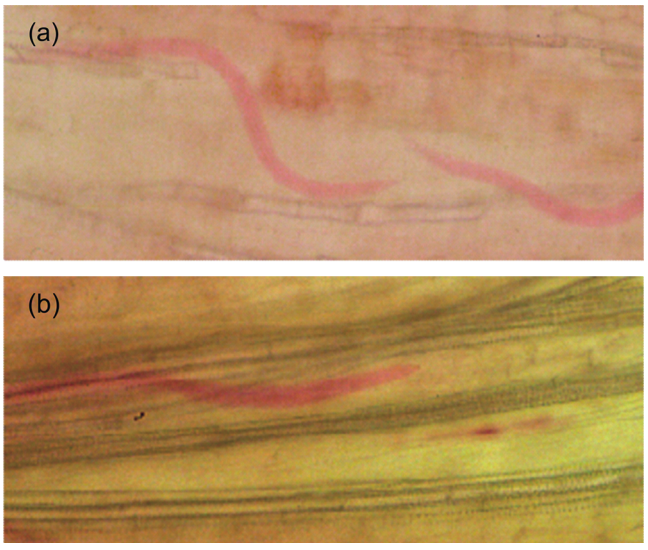

FIG. 2. Neither RKN-resistant DZA045 (A) nor RKN-susceptible F83005 (B) accessions of *Medicago* appear to mount a hypersensitive response to invading *M. incognita* L2. Roots were stained with acid Fuchsin 67 hr after inoculation. Nematodes (stained red) are migrating between cells. Because it is in the vasculature system and slightly swollen, it is likely that the L2 in the susceptible host is beginning to establish a feeding site.

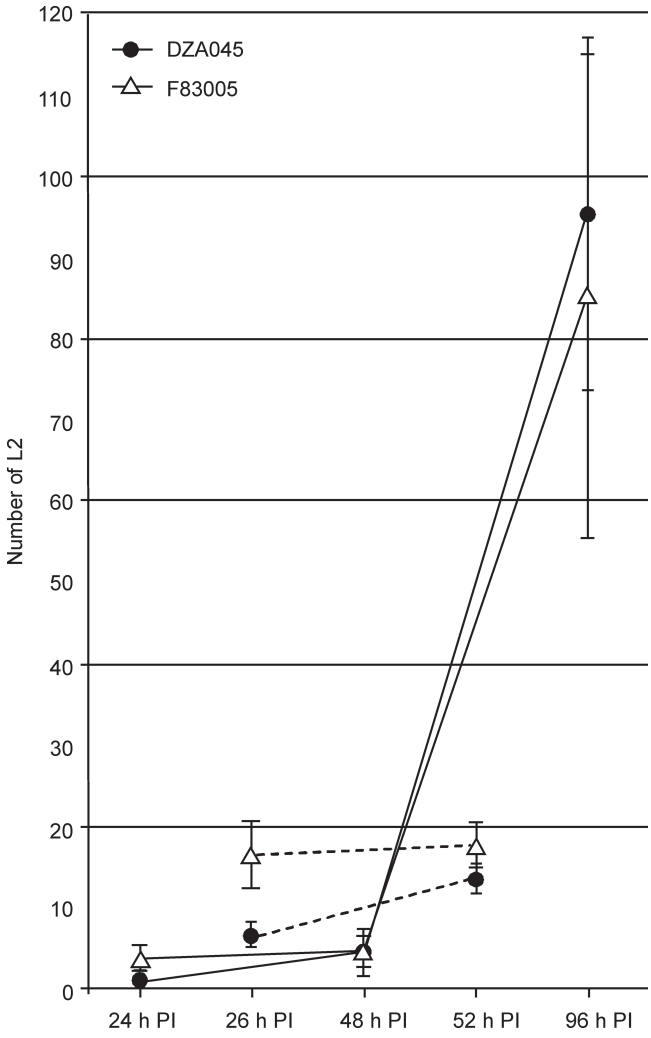

FIG. 3. No difference in penetration by RKN was observed between resistant and susceptible accessions. Number of nematodes within the root-tips of 3-week-old plants inoculated with 1,000 L2 and scored at various times post infection (PI). Error bars represent standard deviation for five replications per time point.

TABLE 3. Twice the numbers of *M. incognita* L2 emigrate from the resistant accession (DZA045) than from the susceptible accession (F83005).

Accession	Number of L2 inoculated/plant	Number of L2 emigrated (±SD)	Em9grated/inoculum (%)
DZA045	500	32 ± 3.4	6.4
DZA045	1,000	69 ± 22.8	6.9
DZA045	2,000	112 ± 27.1	5.6
F83005	500	6.0[a]	1.2
F83005	1,000	25.0[a]	2.5

[a]Data from a single experiment.

TABLE 4. Adult *M. incognita* development on *M. truncatula* accession.

Accession	Inoculum (L2/Plant)	Number of Males/Plant ± S.D.	Males/Inoculum (%)	Number of Females/Plant ± S.D.	Females/Inoculum (%)
DAZ045	500	15.3 ± 2.9	3.06	26.6 ± 18.7	5.32
DZA045	1,000	11.4 ± 8.9	1.14	25.5 ± 23.7	2.55
DZA045	2,000	10.4 ± 1.5	0.04	ND[a]	ND[a]
F83005	1,000	26.4 ± 1.2	2.64	223.0 ± 39.4	22.30

[a]ND = not determined.

RKN develop poorly on the resistant accession: RKN are sexually undifferentiated when they initially infect a host. Although the larvae generally develop as females, stress conditions such crowding or lack of sufficient nutrients may redirect sexual development into males (Triantaphyllou, 1973). Because males are non-feeding and leave the roots at approximately 4 wk post-infection, they are considered to be non-pathogenic. As shown in Table 3, only a few percent of the inoculum developed into males on both resistant and susceptible cultivars, suggesting that DZA045 resistance is not manifested through the redirecting of development towards males. To examine female development, we stained the entire root mass and counted the nematodes in the root (Table 3). Whereas 22% of the initial inoculum developed as females in the susceptible accession, only 3 to 5% developed as females in the resistant accession. The remainder died as L2 inside the root. Collapsed or abnormally developed giant cells were not observed near these dead nematodes, suggesting that they had not established feeding sites before their death (data not shown). Collectively, these results point to a failure of the invading L2 to establish functional feeding sites in the resistant accession DZA045.

Genetic analysis of nematode resistance in DZA045: Of the 177 F6 lines tested, 120 lines showed clockwise coiling (dominant), and 57 lines showed anti-clockwise coiling (recessive). Although this trait is known to be controlled by a single gene, *SPC*, the segregation ratio of F6 did not fit a single gene hypothesis with χ-square analysis. This data suggest that either the F6 lines retain significant heterozygosity or that a bottleneck had occurred during the single-seed descent. Furthermore, in many inbred lines, plants were segregating for resistance. To reduce the effect of heterozygosity in determining the number of genes controlling resistance, we generated F7 lines by selfing the F6 plants. Out of the 143 F7 lines tested, only 36 lines showed the intermediate phenotype (instead of the expected 71 if two genes were involved), suggesting that a single gene controls resistance in DZA045. Although the intermediate phenotypes strongly indicate that the resistance gene is semi-dominant, we did not pursue this area further.

DISCUSSION

Colbran (1958) first reported annual medic as a host for RKN. Emergence of *M. truncatula* as a model to study plant-symbiont interactions, availability of worldwide collections of *M. truncatula* accessions for scientific studies and lack of an incompatible interaction between *Arabidopsis* and RKN or cyst nematodes prompted us to test whether *M. truncatula* could be used as a model to understand plant-nematode interactions. Our results confirm not only that *M. truncatula* is an excellent and typical host for RKN and CLCN, but that substantial natural variability among the accessions in their reaction to infection exists. At the extremes, some accessions were resistant to all species tested (*M. incognita*, *M. arenaria*, *M. hapla* and CLCN), whereas some were susceptible to all. Further, accessions with varying combinations of RKN resistance were also present in the collection.

The mechanism of resistance to RKN in crop plants seems to vary between crop plants and may manifest either pre- or post-infection. Pre-infection resistance, such as is observed in cucumber (Haynes and Jones, 1976) and peanut (Bendezu and Starr, 2003), is due to lack of nematode entry into the plant and is possibly due to the presence of pre-formed chemicals in the plant that are toxic or antagonistic to the nematodes (Huang, 1985). However, in the RKN-resistant *Medicago* accession DZA045, nematode resistance is not due to lack of nematode penetration and is thus more generally similar to resistance in soybean (Herman et al., 1991), alfalfa (Griffin and Elguin, 1977) and tomato (Hadisoeganda and Sasser, 1982), where there is no difference in nematode penetration between the susceptible and resistant lines.

Post-infection resistance mechanisms are manifested after the penetration of the nematode in the host and in some cases are associated with a classical hypersensitive response (HR). The HR is typically explained by the gene-for-gene-model in which an avirulence gene product from the pathogen is specifically recognized by the resistance gene product of the host (Bent, 1996). In contrast, no obvious HR was observed in DZA045, even days after inoculation. The fact that nematodes were still migrating inside the root for several days after penetration suggests that they were unable to establish a feeding site inside the root. Consistent with this observation, we detected significant emigration from the root by larvae, with only 3 to 5% developing as females, leading to net low fecundity.

The number of genes controlling resistance to RKN

seems to differ among hosts and even among varieties. For example, a single gene controls resistance in soybean cultivar 'Forrest' (Luzzi et al., 1994a), whereas multiple genes control resistance in soybean lines PI96354 and PI417444 (Luzzi et al., 1994b). Based on results from screening F7 RIL, it is apparent that a single gene controls resistance in DZA045. Furthermore, mapping suggests that a single gene may control resistance in DZA045 (data not shown).

We screened *Medicago* accessions with SCN, which is considered to have a broad host range (Riggs, 1992). We chose SCN for the study because it is the most economically damaging nematode in the US, and no single soybean cultivar is resistant to all known SCN races (Sipes, 1992). However, our screening results suggest that *M. truncatula* is not a host for SCN. Despite development of a few nematodes from the SCN inbred line, TN2, it is likely that *M. truncatula* is a poor or non-host for SCN. Other annual medics, including *M. hispida*, *M. arabica* and *M. minima*, are also non-hosts for SCN (Riggs, 1992). SBCN, like SCN, has a broad host range and infects a number of economically important crop species (Steele, 1965). However, *M. truncatula* was not a host for this nematode either.

Because *M. truncatula* is not a host for either SCN or SBCN, we tested with CLCN, which is the primary cyst nematode affecting forage legume production in the world (Pederson and Quesenberry, 1998). In fact, *H. trifolii* is the most common cyst nematode in North America, and the economic threshold for this nematode is < 1 egg/ml (Cook and Yeates, 1993). Infection by H. *trifolii* on white clover can reduce the forage production by 14 to 37% (Stelter and Meinl, 1972) and suppresses the amount of nitrogen fixed in the plant (Yeates et al., 1977). Screening *Medicago* accessions with *H. trifolii* identified several accessions that are susceptible and resistant to this nematode. While some accessions were highly susceptible to CLCN, some were mildly susceptible. Accessions that show mild susceptibility to CLCN are still useful, and they can be used to grow in soils that show low initial amounts of inoculum. However, these accessions cannot be used in soils with high nematode inoculum. Accessions that are resistant but poor in their forage yield can be used in breeding programs. Genes conferring resistance to CLCN may be more stable than other nematode resistance genes because CLCN reproduces by obligate mitotic parthenogenesis and therefore it will exhibit lowered genetic variation (Mulvey, 1958; Triantaphyllou and Hirschmann, 1978).

In conclusion, we have established *Medicago truncatula* as a model to understand plant-nematode interactions. Eventual cloning of the nematode resistance gene(s) from the resistant accession should not only help us in understanding the interaction between the host and the nematode, but would help breeders to introgress quickly these gene(s) into superior yet susceptible *Medicago* cultivars. Establishing this model system should provide us with opportunities to identify the mechanism underpinning this unique nematode resistance pathway.

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