Histological Characterization of Resistance to Different Root-Knot Nematode Species Related to Phenolics Accumulation in *Capsicum annuum*

A. Pegard, G. Brizzard, A. Fazari, O. Soucaze, P. Abad, and C. Djian-Caporalino

Institut National de la Recherche Agronomique (INRA), Unit Plant-Microbe Interactions and Plant Health (IPMSV), 400 route des Chappes, Les Templiers, 06903 Sophia Antipolis, France.
Accepted for publication 14 October 2004.

ABSTRACT

Pegard, A., Brizzard, G., Fazari, A., Soucaze, O., Abad, P., and Djian-Caporalino, C. 2005. Histological characterization of resistance to different root-knot nematode species related to phenolics accumulation in *Capsicum annuum*. *Phytopathology* 95:158-165.

In the pepper *Capsicum annuum* CM334, which is used by breeders as a source of resistance to *Phytophthora* spp. and potyviruses, a resistance gene entirely suppresses reproduction of the root-knot nematode (*Meloidogyne* spp.). The current study compared the histological responses of this resistant line and a susceptible cultivar to infection with the three most damaging root-knot nematodes: *M. arenaria*, *M. incognita*, or *M. javanica*. Resistance of CM334 to root-knot nematodes was associated with unidentified factors that limited nematode penetration and with post-

penetration biochemical responses, including the hypersensitive response, which apparently blocked nematode migration and thereby prevented juvenile development and reproduction. High-performance liquid chromatography analysis suggested that phenolic compounds, especially chlorogenic acid, may be involved in CM334 resistance. The response to infection in the resistant line varied with root-knot nematode species and was correlated with nematode behavior and pathogenicity in the susceptible cultivar: nematode species that quickly reached the vascular cylinder and initiated feeding sites in the susceptible cultivar were quickly recognized in CM334 and stopped in the epidermis or cortex. After comparing our data with those from other resistant pepper lines, we suggest that timing of the resistance response and the mechanism of resistance vary with plant genotype, resistance gene, and root-knot nematode species.

The root-knot nematode (*Meloidogyne* spp.) is an important pathogen of several *Solanaceous* crops, especially pepper, potato, and tomato (42,55). Roots attacked by this parasite exhibit characteristic root galls, and infected plants grow poorly or even die because of vascular dysfunction. As sedentary endoparasites, *Meloidogyne* spp. have complex trophic relationships with their host plant and induce specialized feeding structures known as giant cells, which are essential to the nutrition and development of the nematode (7).

Economics, availability, and government regulations have drastically restricted the use of nematicides for controlling plant-parasitic nematodes. Alternatives to nematicides are needed and, among the alternatives, host plant resistance is especially valuable because resistance can provide effective, economical, and environmentally safe nematode control. Unfortunately, large-scale and long-term cultivation of cultivars with single resistance genes may enable root-knot nematodes to overcome that resistance (16,23, 38,46,58). Resistance efficacy and durability, however, may be increased by combining several genes with different resistance mechanisms, as has been demonstrated for resistance to scald in barley (9), rice bacterial blight (58), lentil *Ascochyta* blight (63), and *Pvr4* virus in pepper (11).

Resistance to root-knot nematodes was associated with several dominant genes in the pepper *Capsicum annuum* (19,33). This species of *Solanaceae* is one of the most cultivated vegetable species after tomato in the United States and in developing countries (28). Hendy et al. (33) have shown that two *Capsicum* lines (PM217 and PM687) were resistant to root-knot nematodes and

identified at least four dominant genes. We confirmed their observations and found other dominant genes conferring resistance (19). Some were highly specific, whereas others (*Me*₁ in PM217 and *Me*₃ in PM687) were effective against a wide range of species. One gene, *Me*₃, has been located on the pepper chromosome 9 (20), and localization of the other resistance genes is in progress. Previous comparative histological studies (8,32) showed that the two broad-spectrum resistance genes (*Me*₁ and *Me*₃) induced different response patterns to nematode infection depending on the pepper lines.

In the family *Solanaceae*, resistance usually is associated with a hypersensitive reaction (HR), a rapid and localized cell death in the infected plant in response to pathogen attack. Nematode juveniles surrounded by necrotic cells fail to develop, and they die. The response can occur early and thereby prevent parasite penetration or migration (48). It also can occur later, inhibiting the development of giant cells and suppressing parasite development and multiplication (50). Several authors showed that the HR is locally accompanied by the production or release of highly reactive forms of oxygen, salicylic acid (18), phenolic compounds (4,29,34,39,43,49), or other compounds involved in extracellular signal transduction pathways. Defense gene activation, structural changes (especially cell wall reinforcement), and phytoalexin synthesis (35) also often are observed. These phenomena occur at the infection site within minutes after penetration (29,49).

The inbred pepper line Criollo de Morelos 334 (CM334) displayed a high level of resistance to the three main species of root-knot nematodes: *M. arenaria*, *M. incognita*, and *M. javanica* (19). We named the gene conferring this resistance *Me*₇ (C. Djian-Caporalino, unpublished data). CM334, which originated from the wild population Criollo de Morelos (Mexico) and which produces small and pungent fruit, also has been used in breeding programs as a source of resistance to *Phytophthora capsici* (47) and potyviruses (21).

Corresponding author: C. Djian-Caporalino; E-mail address: caporali@antibes.inra.fr

DOI: 10.1094/PHYTO-95-0158

© 2005 The American Phytopathological Society

The general objectives of the present work were to understand resistance mechanisms to root-knot nematodes in pepper and to find multiple sources or mechanisms so as to enhance resistance durability. Given these general objectives, one specific objective of this article was to quantify nematode penetration by *M. arenaria*, *M. incognita*, and *M. javanica* into the resistant line (CM334) and a susceptible cultivar. A closely related second objective was to compare histological and histochemical responses of the resistant CM334 and a susceptible cultivar to these nematodes. A third objective was to compare and contrast resistance in CM334 with that in two other previously studied sources of nematode resistance in pepper (PM687 and PM217).

MATERIALS AND METHODS

Nematodes. Experiments were conducted with three *Meloidogyne* spp. from the INRA Antibes collection: *M. arenaria* (from Montoux, France), *M. incognita* (from Calissane, France), and *M. javanica* (from Avignon, France). These species were identified using sequence characterized amplified region (SCAR) based polymerase chain reaction (PCR assays) (65). For each species, hatched second-stage juveniles (J2) were collected from infected roots of susceptible tomato plants (cv. Saint Pierre) held in a mist chamber. J2 were stored at 4°C for less than 24 h before use.

Plant material. The pepper cv. Doux Long des Landes (DLL, from a French local population) was used as a susceptible control. It developed galls and allowed nematode reproduction (19). The resistant pepper line CM334 was supplied by A. Palloix (INRA Montfavet, France). It was selfed in insect-proof cages to eliminate outcrossing and was assumed to be homozygous. Experiments were conducted in a growth chamber with a mean temperature of 23°C, a regime of 11 h of light and 13 h of dark, and 60 to 70% relative humidity. Plants were grown individually in small plastic pots containing 75 cm³ of sterilized sand and were randomized within the growth chamber.

Penetration experiment. For each *Meloidogyne* spp., 50 plants of DLL and CM334 were inoculated with a water suspension containing 600 J2. At 1, 2, 3, 4, and 5 days after soil infestation, seedlings were removed from pots and root systems were washed carefully, fixed, and stained with acid fuschin (10). The root system of each plant was examined under a stereo microscope. Penetration was evaluated by counting the stained J2 in the roots. For each combination of pepper genotype and *Meloidogyne* spp., five plants were analyzed on each sampling day. Five additional plants of DLL and CM334 also were inoculated with the same batches of J2, and egg-masses were colored with eosine (19) and counted 6 weeks later. The experiment was repeated.

A two-way analysis of variance (ANOVA) was performed on the data, followed by mean separation with Fisher's protected least significant difference (PLSD, $P = 0.05$) to compare rates of penetration and reproduction. All the statistical work was done using Analyse-it software for Microsoft Excel. Because no significant differences existed between the repeated experiments, data were pooled for analysis.

Plant inoculation and tissue processing for histopathological studies. Four-week-old plants with six to eight expanded leaves were inoculated with 10,000 J2 in 3 ml of aqueous suspension. Nematodes were added at six points around each stem and as close as possible to the root system. Roots were removed from the pots and carefully washed 1, 2, 3, 5, 7, 9, or 14 days after inoculation (DAI). Four plants were used for each combination of pepper line, *Meloidogyne* sp., and time, and the experiment was repeated.

For each plant, five root segments (0.5 to 1.0 cm long) were selected and excised. Root segments were immersed immediately in 1% (vol/vol) glutaraldehyde and 4% (vol/vol) formaldehyde in 100 mM sodium phosphate buffer, pH 7.2, at room temperature and were maintained on a rotary shaker for 3 h. They were rinsed two times with 50 mM sodium phosphate buffer, pH 7.2, for

30 min before dehydration and then dehydrated with agitation in a graded ethanol series (10 to 100%, 10 min at each concentration), followed by two additional steps in 100% ethanol. They were then imbedded in Technovit 7100 (an epoxy resin provided by Kulzer Friedrichsdorf, Germany) according to manufacturer's protocol. Longitudinal sections (3 µm) were mounted on poly-L-lysine loaded slides. One of two glass sides was stained for 4 to 5 min with 0.5% toluidine blue in 0.1 M sodium phosphate buffer, pH 5.5, rinsed with ultra-pure water, and dried. Stained reactions were examined under a Zeiss Axioplan 2 light microscope. Unstained sections of roots were examined and photographed using the light microscope with a UV filter set A2 Zeiss 02 (488002-0000) with 365-nm excitation and 420-nm emission to reveal aromatic compounds. When observed under UV light, yellow and orange autofluorescence revealed the presence of phenolic compounds and a pale blue-autofluorescence revealed the presence of lignified cells (1,52). For each combination (pepper line, *Meloidogyne* sp., and time after inoculation), 10 sections from each of the five different root segments were examined. An average of 400 toluidine blue-stained root sections was observed for each combination of time, *Meloidogyne* sp., and cultivar. For the resistant cv. CM334, ~400 unstained root sections also were observed under UV light.

Identification and chromatographic analysis of phenolic compounds. Because phenolic compounds have been associated with resistance (4,29,34,39,43,49), we quantified the phenolic content of resistant and susceptible pepper roots infected with the three *Meloidogyne* spp. Four-week-old plants of each line were inoculated with 10,000 J2 for each *Meloidogyne* sp. as described for the histological studies. Plants growing in nematode-free sand were used as a control. At 5 DAI, roots were removed from pots and washed carefully in water. Two plants for each combination of pepper line, *Meloidogyne* sp., and the control were analyzed. Inoculated and control fresh root segments (0.4 g) were randomly selected, excised, and placed separately into 10 ml of 100% methanol for 24 h. After roots were removed, 1 ml of each extract was dried under vacuum in a rotary evaporator at room temperature and resuspended in 100 µl of methanol. The concentrated extracts were filtered (0.2 µm) and stored at 4°C, and 20 µl of each was separated and analyzed by high-performance liquid chromatography (HPLC). HPLC separations were performed using an Alltima C18 column and a UV detector coupled to a personal computer and controlled by in-house software. The separation was monitored from 200 to 350 nm. All solvents were of chromatographic grade (Sigma-Aldrich, France). Before use, the HPLC capillary was rinsed with water and methanol. A gradient of methanol prepared in aqueous 0.5% H₃PO₄ made it possible to reliably separate several phenolic compounds. By comparison with standard curves prepared with known phenolic compounds, major peaks were identified and quantified. The experiment was repeated.

RESULTS

Penetration by *Meloidogyne* spp. into roots of susceptible DLL and resistant CM334 cultivars. The nematodes penetrated roots of both pepper lines, but the two-way ANOVA revealed a significant influence of plant genotype ($P < 0.0001$), *Meloidogyne* sp. ($P < 0.0001$ to $P = 0.02$), and time after inoculation ($P < 0.0001$). By 3 DAI, significantly more ($P < 0.0001$) J2 had penetrated the susceptible cultivar than the resistant line (Table 1). This difference increased substantially with time. At 5 DAI, the maximum number of J2 had penetrated. Whereas 29 to 43% of the inoculum penetrated the roots of the susceptible pepper, <4% penetrated the roots of CM334. Significant differences ($P = 0.0001$ to $P = 0.0031$) in the ability to invade the roots also occurred among *Meloidogyne* spp. by 2 or 3 DAI. More J2 of *M. incognita* than of *M. javanica* or *M. arenaria* penetrated the roots of

DLL and CM334. In DLL, egg masses were produced by 27, 23, and 18% of J2 inoculum for *M. incognita*, *M. javanica*, and *M. arenaria*, respectively. In CM334, however, no egg masses were detected after 6 weeks.

Histological observations of susceptible DLL roots. Examination of approximately 8,400 susceptible root sections showed that, although all three nematode species were able to penetrate roots, migrate, and develop normally (Fig. 1), they differed in time required to reach the vascular cylinder and to develop giant cells. Observation of numerous sections indicated that the differences were constant under the experimental conditions. Most of the J2 entered the roots and moved intercellularly through the root tip and elongation zone (Fig. 1A and E). Several J2 invaded the same site (especially *M. incognita*) and migrated in groups through the cortex (Fig. 1I). The first cellular changes appeared 3 DAI for *M. arenaria* and *M. javanica* (Fig. 1B), and 5 DAI for *M. incognita* (Fig. 1J). At 5 DAI, giant cells contained two nuclei for *M. incognita* (Fig. 1J) and four or more nuclei for *M. arenaria* or *M. javanica* (Fig. 1C and F). Giant cells containing multiple nuclei and juveniles molted to stage 3 were seen 7 DAI for *M. arenaria* and *M. javanica* (Fig. 1G), and 9 DAI for *M. incognita* (Fig. 1K). Giant cells reaching the maximum length of 400 μ m were seen 9 DAI for *M. arenaria* and *M. javanica* (Fig. 1D and H) and 14 DAI for *M. incognita* (Fig. 1L). Six weeks after inoculation, galls were 2 mm in diameter for all three species in the susceptible line.

Histological observations of resistant CM334 roots. Examination of approximately 16,800 resistant root sections showed that, among the few J2 penetrating the roots for the three species, all remained clustered at the root apex and died without development (i.e., no J2 was found in the vascular stele; J2 were found in the epidermis or cortex only) and there was no formation of giant cells. Moreover, the J2 were surrounded by necrotic cells. The lesions, which were green when stained with toluidine blue, were first discrete and highly localized around the nematode head (Fig. 2A, E, and I). During migration, nematodes left a patch of necrotic cells which usually was parallel to the root axis (Fig. 2F and L) and which stained more intensely with time.

When resistant root sections were examined under UV light, a yellow or orange autofluorescence characteristic of aromatic compounds was seen within the cells and in the cell walls. This fluorescence appeared early, became intensely bright orange 3 DAI, and only occurred in epidermic and cortical cells around the nematodes. This reaction was observed as early as 1 DAI in response to *M. arenaria*, 1 or 2 DAI in response to *M. javanica*, or 3 DAI in response to *M. incognita* (Fig. 2A, E, and J). Similar fluorescence was not observed in the susceptible roots and also was absent before infection in the resistant roots (data not shown).

At 1 or 2 DAI with *M. arenaria* or *M. javanica*, and 5 DAI with *M. incognita*, yellow or orange autofluorescence was detected in intercellular spaces around the nematode (Fig. 2A, E, and K). For *M. incognita*, it was intracellular 3 DAI, but not in the intercellu-

lar space (Fig. 2J). Substantial necrosis also occurred at the root apex when multiple J2 had penetrated (Fig. 2J and K).

In CM334 cellular necrosis differed for the three *Meloidogyne* spp. For *M. arenaria*, necrosis was concentrated in the epidermis (the head of the J2 was surrounded by necrotic cells) (Fig. 2A) and outer cortex (Fig. 2B and 2C). In contrast, necrosis occurred in the outer and intermediate cortex for *M. javanica* (Fig. 2E, F, G, and H) and in the whole cortex for *M. incognita* (Fig. 2I, J, K, and L). Necrosis occurred shortly after penetration for *M. arenaria* (Fig. 2A) and *M. javanica*, but later for *M. incognita* (Fig. 2J).

Signs of nematode death were first visible 7 DAI for *M. arenaria* or *M. javanica* and 9 DAI for *M. incognita* (Fig. 2D, G, and H). Because necrotic cells of both nematode and adjacent root exhibited a darkening of the orange fluorescence, visualizing the nematode was difficult (Fig. 2L). Fluorescence increased on thickened cell walls and in intercellular spaces near the nematode.

No necrosis occurred on roots of the susceptible cultivar. Although no yellow or orange autofluorescence was detected in susceptible roots, vessel cell walls of the resistant line and susceptible cultivar exhibited a pale blue-white autofluorescence under UV light. A pale blue-white autofluorescence also was observed in walls of some necrotic cells 7 or 9 DAI of resistant roots (Fig. 2D, H, and L).

HPLC analysis of phenolic compounds. Phenolic compounds were extracted from noninoculated and inoculated susceptible and resistant roots and analyzed by HPLC. Results were similar for all three nematode species and, to simplify the presentation, only data for *M. arenaria* at 254 nm are shown (Fig. 3).

Based on comparison with a standard curve prepared with known phenolic compounds, a major peak was identified as chlorogenic acid (retention time: 26.6 min) in extracts of inoculated roots of the resistant CM334 line. Amounts of chlorogenic acid differed between extracts of noninoculated and inoculated roots (sampled 5 DAI) within each pepper cultivar (Table 2). Inoculated CM334 roots contained 59 times more chlorogenic acid per gram of root tissue than noninoculated CM334, and 7 times more than inoculated DLL.

An additional specific peak only present in inoculated CM334 roots (retention time: 34 min) was unidentified. This phenolic compound was eight times less concentrated than the chlorogenic acid.

DISCUSSION

The first aims of this study were to compare root-knot nematode penetration and subsequent development in a resistant pepper line and a susceptible pepper cultivar. We showed that the three different *Meloidogyne* spp. were able to penetrate the roots of both pepper genotypes but that penetration differed among the nematode species and was much reduced in the resistant line. Nematode development after penetration also was greatly affected

TABLE 1. Number of nematodes in roots and egg masses on roots of the cv. Doux Long des Landes (DLL; susceptible) and CM334 (resistant) pepper cultivars inoculated with 600 *Meloidogyne* second-stage juveniles (J2)^z

Time after inoculation	Susceptible DLL			Resistant CM334		
	<i>M. incognita</i>	<i>M. javanica</i>	<i>M. arenaria</i>	<i>M. incognita</i>	<i>M. javanica</i>	<i>M. arenaria</i>
No. of J2 per root system						
1 day	36 $\pm$ 6 a	15 $\pm$ 4 bc	21 $\pm$ 3 ab	8 $\pm$ 2 c	6 $\pm$ 1 c	6 $\pm$ 1 c
2 days	46 $\pm$ 7 a	24 $\pm$ 3 b	20 $\pm$ 3 b	19 $\pm$ 4 bc	7 $\pm$ 1 c	7 $\pm$ 1 c
3 days	82 $\pm$ 6 a	56 $\pm$ 3 b	55 $\pm$ 3 b	22 $\pm$ 4 c	9 $\pm$ 2 d	9 $\pm$ 1 d
4 days	229 $\pm$ 14 a	163 $\pm$ 9 b	170 $\pm$ 8 b	19 $\pm$ 3 c	4 $\pm$ 1 d	3 $\pm$ 1 d
5 days	255 $\pm$ 11 a	184 $\pm$ 11 b	173 $\pm$ 15 b	18 $\pm$ 2 c	7 $\pm$ 2 cd	6 $\pm$ 1 d
No. of egg-masses per root system						
6 weeks	160 $\pm$ 14 a	136 $\pm$ 10 ab	107 $\pm$ 11 b	0 c	0 c	0 c

^z Values are the means ($\pm$ one standard error) of 10 replicate plants. In a row, values followed by the same letter are not significantly different according to Fisher's protected least significant difference at $P = 0.05$. DAI = days after inoculation.

by host genotype and nematode species. Our data suggest that CM334 pepper has at least two kinds of resistance to *Meloidogyne* spp. One kind of resistance suppresses penetration by J2 and the other kind blocks development after penetration.

Plant resistance can occur at several levels. Failure of J2 to penetrate roots of resistant CM334 (2% of penetration in resistant roots compared with 30% in susceptible roots) may indicate physical root barriers. Such barriers were suggested previously for resistant grape rootstocks (2,27), a cotton cultivar (3), and a soybean cultivar (22). It also may indicate that the roots did not attract or even repelled J2, or that J2 penetrated and then left the

roots. Such protection was shown for *Cucumis sativus*: a triterpene, cucurbitacin, that repelled *Meloidogyne* J2 was isolated from root exudates (31). Similarly, amino acids exuded from *Sesamum indicum* roots have a nematostatic effect on *Meloidogyne* J2 (59).

Moreover, in the resistant line, J2 remained vermiform and clustered in cortical cells at the root apex. No galling occurred and no egg mass developed. This suggests that CM334 has very active post-penetration biochemical defense mechanisms which blocked nematode migration, development, and reproduction. The post-penetration response of the incompatible CM334 roots was

Fig. 1. Longitudinal sections through susceptible cv. Doux Long des Landes roots inoculated with **A to D**, *Meloidogyne arenaria* (Ma), **E to H**, *M. javanica* (Mj), and **I to L**, *M. incognita* (Mi). Sections were stained with toluidine blue. DAI = days after inoculation, ne = nematode, ep = epidermis, co = cortex, vc = vascular cylinder, gc = giant cells. Bars = 100 μ m.

considered a classical HR. This HR occurred more quickly than did the susceptible response.

As reported by others for different plants (30,39,40,48,57,61), the HR in CM334 involved substantial necrosis and it seems

possible, although not yet proven, that the necrosis limits nematode development.

Among molecules that may actively contribute to plant defense against nematodes and other pathogens, phenolics are often cited

Fig. 2. Longitudinal sections through resistant CM334 roots inoculated with **A to D**, *Meloidogyne arenaria* (Ma), **E to H**, *M. javanica* (Mj), and **I to L**, *M. incognita* (Mi). Sections were unstained and observed with UV light, except that sections in reduced images were stained with toluidine blue. DAI = days after inoculation, ne = nematode, ep = epidermis, co = cortex, HR = hypersensitive reaction, vc = vascular cylinder. Bars = 100 μm.

(4,6,29,34,39,43,44,49). In the current study, histological analysis, UV light observations, and HPLC analysis of noninfected and infected pepper roots showed an association between phenolics and resistance to root-knot nematodes. In fact, these compounds accumulated locally inside cells close to the migrating nematode early in injured tissues of the resistant line only. Phenolic compounds were concentrated in a small number of cells and in the intercellular spaces of root tissue around J2 when observed under UV light. We identified chlorogenic acid as the major phenolic compound in CM334 pepper roots penetrated by nematodes. Chlorogenic acid is known to affect nematode coordination (34). The presence of this compound may cause the browning and the resistant reaction of both *Nicotiana repanda* (43) and *Nemared* tomato (34,49) to *M. incognita*. In vivo activity of chlorogenic acid could be due to the quinic acid or caffeic acid components which are released by the action of hydrolytic enzymes such as esterases (14). The oxidized forms of phenolic compounds, which also occurred in high concentrations in roots of resistant tomato plants, might contribute to the *M. incognita* resistance (4) by creating a toxic environment for nematode penetration and multiplication. This also was proposed for fungi (5) and bacteria (45). Because they can be detected with UV light, histochemical procedures, or HPLC, phenolics may be useful for identifying additional resistant cultivars.

In related work, we identified several genes responsible for resistance to *Meloidogyne* spp. in different *Capsicum annuum* lines (PM217, PM687, and CM334) and in cv. Yolo Wonder (YW) (19,33). The timing, location, and nature of the resistant reaction appear to vary depending on the plant genotype, the particular resistance gene, and the *Meloidogyne* sp. (Table 3). There was immediate cellular necrosis in the root epidermis adjacent to the J2 (8,32) (sometimes only the head had penetrated the root epidermis) in response to *M. incognita* in PM687 (*Me*₃ gene) (8,32),

M. arenaria in PM217 (*Me*₁ gene) (32), and in CM334 (*Me*₇ gene), as reported with the *Mi*_{-1,2} gene in resistant tomato plants (48). Necrosis was observed in the intermediate cortex 1 or 2 DAI in PM217 (*Me*₁ gene) (32), PM687 (*Me*₃ gene) (32), and CM334 (*Me*₇ gene) with *M. javanica*. Cellular necrosis was visible only 3 DAI for *M. incognita* and was concentrated in the inner cortex near the vascular cylinder in CM334 (*Me*₇ gene). Small giant cells with few gathered nuclei began to develop within 4 DAI in resistant pepper PM217 (*Me*₁ gene) with *M. incognita*, PM687 (*Me*₃ gene) with *M. arenaria*, and YW (*Me*₅ gene) with *M. javanica* (8,32) but necrosis was not evident until 5 DAI and females were unable to reproduce (15,24,26,51). A similar degradation of the feeding site was described for resistant lines of other plants, such as sugar beet, oilseed radish, potato, and soybean (8,25,53,62,64).

Biotypes of *Meloidogyne* spp. that are able to overcome resistance have been identified (36,60). In artificial selection experiments, an *M. incognita* lineage virulent on pepper plants carrying the *Me*₃ gene was obtained; however, in spite of the drastic selection pressure, the nematode appeared unable to overcome the *Me*₁ gene (13). Researchers have proposed that virulence could result from the nematode's ability to overcome the early HR occurring in the outer tissues of the root (i.e., the epidermis and root cortex)

TABLE 2. Quantities of chlorogenic acid in susceptible or resistant pepper roots infected or not infected with *Meloidogyne arenaria*^a

Roots	Chlorogenic acid (μg) per gram of root tissue	
	Susceptible DLL	Resistant CM334
Noninfected	67.4 ± 8.4	9.6 ± 0.3
Infected	77.9 ± 3.1	569.6 ± 62.3

^a DLL = cv. Doux Long des Landes. Values are the means (± one standard error) of four replicate root systems.

Fig. 3. High performance liquid chromatography spectrum at 254 nm of extracts from cv. Doux Long des Landes (DLL; susceptible) and CM334 (resistant) pepper roots. DLL **A**, noninoculated and **B**, inoculated with *M. arenaria*. CM334 **C**, noninoculated and **D**, inoculated with *M. arenaria*.

TABLE 3. Histopathological reactions to *Meloidogyne* spp. depending on pepper genotype, resistance gene (R), and nematode species^z

Plant genotype (R)	<i>M. arenaria</i>	<i>M. javanica</i>	<i>M. incognita</i>
CM334 (<i>Me</i> ₇)	Immediate necrosis in epidermis 1 DAI	Immediate necrosis in cortex 1 or 2 DAI	Necrosis in cortex 3 DAI
PM217* (<i>Me</i> ₁)	Immediate necrosis in epidermis 1 DAI	Rapid necrosis in cortex 2 DAI	Small giant cells, necrosis not evident until 5 DAI
PM687* (<i>Me</i> ₃)	Rapid necrosis in cortex and small giant cells with necrosis 5 DAI	Rapid necrosis in cortex 2 DAI	Immediate necrosis in epidermis 1 DAI
Yolo Wonder* (<i>Me</i> ₅)	Normal giant cells	Small giant cells, necrosis not evident until 5 DAI	Normal giant cells
Susceptible DLL	Normal giant cells with first cellular changes 3 DAI	Normal giant cells with first cellular changes 3 DAI	Normal giant cells with first cellular changes 5 DAI

^z DAI = days after inoculation; * indicates results from Hendy et al. (28); DLL = cv. Doux Long des Landes.

(8,12). On the other hand, defense reactions elicited deeper in the root (i.e., close to or within the vascular cylinder) would irreversibly block any further nematode development, thus preventing the selection of a virulent genotype. This hypothesis could be tested by experiments with CM334 and the three *Meloidogyne* spp. We predict that attempts to select for *M. arenaria* that is virulent on CM334 would succeed (because HR occurs early and in outer tissues), whereas similar attempts with *M. incognita* would fail (because HR occurs late and in inner tissues). The response of the resistant line to the three nematode species seemed to be correlated with the behavior and pathogenicity of the three species on the susceptible cultivar. Those species (*M. arenaria* and *M. javanica*) that rapidly moved to the vascular cylinder and rapidly initiated giant cells in the susceptible cultivar were quickly recognized and immobilized in the epidermis or cortex of the resistant line. In contrast, *M. incognita* required more time to establish feeding sites in the susceptible cultivar, more J2 penetrated the roots of DLL and CM334, and apparently this species was not recognized or immobilized by the resistant line until after the J2 had moved from the cortex and into the vascular cylinder. Perhaps those pathogenicity factors, including proteins secreted by the amphids (56) or stylet (17,37,54), that account for establishment of feeding sites in susceptible plants also trigger defense mechanisms in resistant plants.

Our results suggest diverse mechanisms of resistance in the roots of a nematode-resistant pepper line. We showed that the resistance response varies depending on the host and nematode genotypes, as in fungal systems (41), and that induction of phenolic compounds such as chlorogenic acid may be an important component of resistance in the CM334 line. In addition, our histological analyses of a *C. annuum*-resistant line provide relevant information about the spatial relationship between host and pathogen; the timing of nematode penetration, nematode infection, and host response; and how the resistance response varies with different *Meloidogyne* spp. We also showed that the response to different root-knot nematodes in susceptible and resistance plants was correlated (i.e., the more quickly a nematode species established feeding sites in the susceptible host, the more quickly it was recognized and immobilized in the resistant host).

ACKNOWLEDGMENTS

We thank M. Nicole for helpful discussions, J. de Almeida Engler for critically reading the manuscript, L. Pijarowski for helping in the bibliographic study, P. Lecomte for technical assistance in histological experiments, and M. Ponchet and L. Lapeyre for assistance in HPLC analysis.

LITERATURE CITED

- Andary, C., Mondolot-Cosson, L., and Dai, G. H. 1996. *In situ* detection of polyphenols in plant-microorganism interactions. Pages 45-53 in: Histology, Ultrastructure and Molecular Cytology of Plant-Microorganism Interactions. M. Nicole and V. Gianinazzi-Pearson, eds. Kluwer Academic, Dordrecht, the Netherlands.
- Anwar, S. A., and McKenry, M. V. 2000. Penetration, development and reproduction of *Meloidogyne arenaria* on two new resistant *Vitis* spp. *Nematropica* 30:9-17.
- Anwar, S. A., Trudgill, D. L., and Philipps, M. S. 1994. The contribution of variation in invasion and development rates of *Meloidogyne incognita* to host status differences. *Nematologica* 40:579-586.
- Bajaj, K. L., and Mahajan, R. 1977. Phenolic compounds in tomato susceptible and resistant to *Meloidogyne incognita* (Kofoid et White). *Chitwood. Nematol. Mediterr.* 5:329-333.
- Baker, C. J., and Orlando, B. W. 1995. Active oxygen in plant pathogenesis. *Annu. Rev. Phytopathol.* 33:299-321.
- Bingefors, S. 1982. Nature of inherited nematode resistance in plants. Pages 187-219 in: Pathogens Vectors and Plant Diseases: Approaches to Control. K. F. Harris and K. Marmorosh, eds. Academic Press, New York.
- Bird, A. F. 1961. The ultrastructure and histochemistry of a nematode-induced giant cell. *J. Cell. Biol.* 11:701-715.
- Bleve-Zaccheo, T., Bongiovanni, M., Melillo, M. T., and Castagnone-Sereno, P. 1998. The pepper resistance genes *Me1* et *Me3* induce differential penetration rates and temporal sequences of root cell ultrastructural changes upon nematode infection. *Plant Sci.* 133:79-90.
- Brown, A. H. D., Garvin, D. F., Burdon, J. J., Abbott, D. C., and Read, B. J. 1996. The effect of combining scald resistance genes on disease levels, yield and quality traits in barley. *Theor. Appl. Genet.* 93:361-366.
- Byrd, D. W., Kirkpatrick, T., and Barker, K. R. 1983. An improved technique for clearing and staining plant tissues for detection of nematodes. *J. Nematol.* 15:142-143.
- Caranta, C., Thabuis, A., and Palloix, A. 1999. Development of a CAPS marker for the Pvr4 locus: A tool for pyramiding potyvirus resistance genes in pepper. *Genome* 42:1111-1116.
- Castagnone-Sereno, P., Bongiovanni, M., and Djian-Caporalino, C. 2001. New data on the specificity of the root-knot nematode resistance genes *Me1* and *Me3* in pepper. *Plant Breed.* 120:429-433.
- Castagnone-Sereno, P., Bongiovanni, M., Palloix, A., and Dalmasso, A. 1996. Selection for *Meloidogyne incognita* virulence against resistance genes from tomato and pepper and specificity of the virulence/resistance determinants. *Eur. J. Plant Pathol.* 102:585-590.
- Clark, R. S., Kuc, J., Henze, R. E., and Quacembush, F. W. 1959. The nature and fungitoxicity of an amino acid addition product of chlorogenic acid. *Phytopathology* 49:594-598.
- Cotton, J., and Hayes, J. D. 1969. Genetic resistance to the cereal cyst nematode (*Heterodera avenae*). *Heredity* 24:596-601.
- Dautova, M., and Gommers, F. J. 2000. *Meloidogyne* spp. in Macedonia: Distribution and virulence for *Mi* resistance in tomato. *Nematol. Mediterr.* 28:121-128.
- Davis, E. L., Hussey, R. S., Baum, T. J., Bakker, J., Schots, A., Rosso, M. N., and Abad, P. 2000. Nematode parasitism genes. *Annu. Rev. Phytopathol.* 38:365-396.
- Dixon, R. A., and Harisson, M. J. 1994. Early events in the activation of plant defence responses. *Annu. Rev. Phytopathol.* 32:479-501.
- Djian-Caporalino, C., Pijarowski, L., Fazari, A., Samson, M., Gaveau, L., O'Byrne, C., Lefebvre, V., Caranta, C., Palloix, A., and Abad, P. 2001. High-resolution genetic mapping of the pepper (*Capsicum annuum* L.) resistance loci *Me3* and *Me4* conferring heat-stable resistance to root-knot nematodes (*Meloidogyne* spp.). *Theor. Appl. Genet.* 103:592-600.
- Djian-Caporalino, C., Pijarowski, L., Januel, A., Lefebvre, V., Daubeze, A., Palloix, A., Dalmasso, A., and Abad, P. 1999. Spectrum of resistance to root-knot nematodes and inheritance of heat-stable resistance in pepper (*Capsicum annuum* L.). *Theor. Appl. Genet.* 99:496-502.
- Dogimont, C., Palloix, A., Daubeze, A. M., Marchoux, G., Gebre-Selassie, K., and Pochard, E. 1996. Genetic analysis to broad spectrum resistance to potyviruses using doubled haploid lines of pepper (*Capsicum annuum*). *Euphytica* 88:231-239.
- Dropkin, V. H., and Nelson, P. E. 1960. The histopathology of root-knot nematode infections in soybeans. *Phytopathology* 50:442-447.

23. Eddaoudi, M., Ammati, M., and Rammah, A. 1997. Identification of the resistance breaking populations of *Meloidogyne* on tomatoes in Morocco and their effect on new sources of resistance. *Fundam. Appl. Nematol.* 20:285-289.
24. Endo, B. Y. 1965. Histological responses of resistant and susceptible soybean varieties and backcross progeny to entry and development of *Heterodera glycines*. *Phytopathology* 53:375-381.
25. Endo, B. Y. 1991. Ultrastructure of initial response of susceptible and resistant soybean roots to infection by *Heterodera glycines*. *Rev. Nematol.* 14:73-94.
26. Fassuliotis, G., Deakin, J. R., and Hoffman, J. C., 1970. Root-knot nematode resistance in snap beans: Breeding and nature of resistance. *J. Am. Soc. Hortic. Sci.* 95:640-645.
27. Ferris, H. S., Schneider, S. M., and Stuth, M. C. 1982. Probability of penetration and infection by root-knot nematode *Meloidogyne arenaria*, in grape cultivars. *Am. J. Enol. Viticult.* 33:31-35.
28. Fery, R. L., and Thies, J. A. 1997. Evaluation of *Capsicum chinense* Jacq. cultigens for resistance to the southern root-knot nematode. *Hortscience* 32:923-926.
29. Giebel, J. 1970. Phenolic content in roots of some *Solanaceae* and its influence on IAA-oxidase activity as an indicator of resistance to *Heterodera rostochiensis*. *Nematologica* 16:22-32.
30. Grundler, F. M. W., Sobezak, M., and Lange, S. 1997. Defense responses of *Arabidopsis thaliana* during invasion and feeding site induction by the plant-parasitic nematode *Heterodera glycines*. *Physiol. Mol. Plant Pathol.* 50:419-429.
31. Hayne, R. L., and Jones, C. M. 1976. Effects of the *Bi* locus in cucumber on reproduction, attraction, and response of the plant to the southern root-knot nematode. *J. Am. Soc. Hortic. Sci.* 101:422-424.
32. Hendy, H., Dalmasso, A., and Cardin, M. C. 1985. Differences in resistant *Capsicum annuum* attacked by different *Meloidogyne* species. *Nematologica* 31:72-78.
33. Hendy, H., Pochard, E., and Dalmasso, A. 1983. Identification de 2 nouvelles sources de résistance aux nématodes à galles chez le piment. Note présentée par P. Pécaut. *C.R. Acad. Agric. France* 11:817-822.
34. Huang, C. L., and Rhode, R. A. 1973. Phenol accumulation related to resistance in tomato to infection by root-knot and lesion nematodes. *J. Nematol.* 5:253-258.
35. Hutcheson, S. W. 1998. Current concepts of active defense in plants. *Annu. Rev. Phytopathol.* 36:59-90.
36. Jarquin-Barberena, H., Dalmasso, A., de Guiran, G., and Cardin, M. 1991. Acquired virulence in the plant parasitic nematode *Meloidogyne incognita*. *Rev. Nématol.* 14:261-275.
37. Jaubert, S., Laffaire, J.-B., Abad, P., and Rosso, M. N. 2002. A polygalacturonase of animal origin isolated from the root-knot nematode *Meloidogyne incognita*. *FEBS Lett.* 522:109-112.
38. Kaloshian, I., Yaghoobi, J., Liharska, T., Hanson, D., Hontelez, J., Hogan, P., Jesse, T., Wijbrandi, J., Simons, G., Zabel, P., and Williamson, V. M. 1998. Genetic and physical localization of the root-knot nematode resistance locus *Mi* in tomato. *Mol. Gen. Genet.* 257:376-385.
39. Kaplan, D. T. 1978. Characterization of soybean incompatibility to *Meloidogyne incognita* and its association to glyceollin accumulation in infected root tissue. Ph.D. diss. University of California, Riverside.
40. Keen, N. T., and Bruegger, B. B. 1977. Phytoalexins and chemicals that elicit their production in plants. Pages 1-26 in: *Host Plant Resistance to Pests*. P. Hedin, ed. ACS Symp. Ser. Am. Chem. Soc. Ser. No. 62.
41. Keen, N. T., and Littlefield, L. H. 1979. The possible association of phytoalexins with resistance gene expression in flax to *Melampsora lini*. *Physiol. Plant. Pathol.* 14:265-280.
42. Khan, M. W., and Haider, S. H. 1991. Comparative damage potential and reproduction efficiency of *Meloidogyne javanica* and races of *M. incognita* on tomato and eggplant. *Nematologica* 37:293-303.
43. Milne, D. L., Boshoff, D. N., and Buchan, P. W. W. 1965. The nature of resistance of *Nicotiana repanda* to the root-knot nematode, *Meloidogyne javanica*. *S. Afr. Agric. Sci.* 8:557-567.
44. Nicholson, R. L., and Hammerschmidt, R. 1992. Phenolic compounds and their role in disease resistance. *Annu. Rev. Phytopathol.* 30:369-389.
45. Novacky, A., Pike, S. M., and Popham, P. L. 1993. Varying membrane properties of cells in tissues inoculated with HR-inducing bacteria. Pages 75-93 in: *Biotechnology and Plant Protection, Bacterial Pathogenesis and Disease Resistance*. D. Bills and S. D. Kung, eds. Butterworth, Guildford, UK.
46. Omat, C., Verdejo-Lucas, S., and Sorribas, F. J. 2001. A population of *Meloidogyne javanica* in Spain virulent to the *Mi* resistance gene in tomato. *Plant Dis.* 85:271-276.
47. Palloix, A., Daubeze, A. M., Phaly, T., and Pochard, E. 1990. Breeding transgressive lines of pepper for resistance to *Phytophthora capsici* in a recurrent selection system. *Euphytica* 51:141-150.
48. Paulson, R. E., and Webster, J. M. 1972. Ultrastructure of the hypersensitive reaction in roots of tomato, *Lycopersicon esculentum* L., to infection by the root-knot nematode, *Meloidogyne incognita*. *Physiol. Plant Pathol.* 2:227-234.
49. Pi, C. L., and Rhode, R. A. 1967. Phenolic compounds and host reaction in tomato to injury caused by root knot and lesion nematodes. (Abstr.) *Phytopathology* 57:344.
50. Pontier, D., Gan, S. S., Amasino, R. M., Roby, D., and Larn, E. 1999. Markers for hypersensitive response and senescence show distinct patterns of expression. *Plant Mol. Biol.* 39:1243-1255.
51. Powell, N. T. 1962. Histological basis of resistance to root-knot nematodes in flue-cured tobacco. (Abstr.) *Phytopathology* 52:25.
52. Ribereau-Gayon, P. 1968. Les composés phénoliques des végétaux, Dunod, ed. Paris.
53. Rice, S. L., Leadbeater, B. S. C., and Stone, A. R. 1985. Changes in cell structure in roots of resistant potatoes parasitized by potato cyst-nematodes: Potatoes with resistance gene *H1* derived from *Solanum tuberosum* spp. *andigena*. *Physiol. Plant Pathol.* 27:219-234.
54. Rosso, M. N., Favory, B., Pirotte, C., Arthaud, L., de Boer, J. M., Hussey, R. S., Bakker, J., Baum, T. J., and Abad, P. 1999. Isolation of a cDNA encoding a β -1,4-endoglucanase in the root-knot nematode *Meloidogyne incognita* and expression analysis during plant parasitism. *Mol. Plant-Microbe Interact.* 12:585-591.
55. Sasser, J. N. 1977. Worldwide dissemination and importance of the root-knot nematode, *Meloidogyne* spp. *J. Nematol.* 22:585-589.
56. Semblat, J. P., Rosso, M. N., Hussey, R. S., Abad, P., and Castagnone-Sereni, P. 2001. Molecular cloning of a cDNA encoding an amphid-secreted putative avirulence protein from the root-knot nematode *Meloidogyne incognita*. *Mol. Plant-Microbe Interact.* 14:72-79.
57. Sillero, J. C., and Rubiales, D. 2002. Histological characterization of resistance to *Uromyces viciae-fabae* in faba bean. *Phytopathology* 92:294-299.
58. Singh, S., Sidhu, J. S., Huang, N., Vikal, Y., Li, Z., Brar, D. S., Dhaliwal, H. S., and Khush, G. S. 2001. Pyramiding three bacterial blight resistance genes (*Xa5*, *Xa13* and *Xa21*) using marker-assisted selection into indica rice cultivar PR106. *Theor. Appl. Genet.* 102:1011-1015.
59. Tanda, A. S., Atwal, A. S., and Bajaj, Y. P. S. 1989. *In vitro* inhibition of root-knot nematode *Meloidogyne incognita* by Sesame root-exudate and its amino acids. *Nematologica* 35:115-124.
60. Tzortzakakis, E. A., and Gowen, S. R. 1996. Occurrence of a resistance breaking pathotype of *Meloidogyne javanica* on tomatoes in Crete, Greece. *Fundam. Appl. Nematol.* 19:238-288.
61. Valette, C., Andary, C., Geiger, J. P., Sarah, J. L., and Nicole, M. 1998. Histochemical and cytochemical investigations of phenols in roots of banana infected by the burrowing nematode *Radopholus similis*. *Phytopathology* 88:1141-1148.
62. Wyss, U., Grundler, F. M. W., and Munch, A. M. 1992. The parasitic behaviour of second-stage juveniles of *Meloidogyne incognita* in roots of *Arabidopsis thaliana*. *Nematologica* 38:98-111.
63. Ye, G., McNeil, D. L., and Hill, G. D. 2002. Breeding for resistance to lentil *Ascochyta* blight. *Plant Breed.* 121:185-191.
64. Yu, M. H., and Steele, A. E. 1981. Host-parasite interaction of resistant sugar beet and *Heterodera schachtii*. *J. Nematol.* 13:206-212.
65. Zijlstra, C., Donkers-Venne, T. H. M., and Fargette, M. 2000. Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterised amplified region (SCAR) based PCR assays. *Nematology* 2:847-853.