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Host-pathogen interaction of root-knot nematode *Meloidogyne incognita* on pepper in the southeast of Spain

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Abstract The feeder roots of pepper plants (cv. California Wonder) in Campo de Cartagena (southeast Spain) were found to be severely infected by *Meloidogyne incognita*. Morphometric traits, differential host test and DNA analysis based on PCR were used to characterize the nematode. Naturally and artificially infected pepper plants showed severe yellowing and stunting, with heavily deformed and damaged root systems. Root galls were spherical and commonly contained more than one female and egg masses with eggs. Typical giant cells with a granular cytoplasm and many hypertrophied nuclei were observed in histological preparations. The relationship between initial nematode population density (*Pi*) and pepper plant growth was tested in greenhouse experiments with inoculum levels that varied from 0 to 64 eggs and second-stage juveniles (J_2) ml^{-1} soil. A Seinhorst model was fitted to plant height and top fresh weight data of inoculated and non-inoculated plants. The tolerance limit with respect to plant height and fresh top weight of pepper to *M. incognita* was estimated as 0.85 eggs and $J_2 ml^{-1}$ soil. The minimum relative values (*m*) for plant height and top fresh weight were 0.15 and 0.16, respectively, at $Pi \geq 64$ eggs and $J_2 ml^{-1}$ soil. The maximum nematode reproduction rate (*Pf/Pi*) was 315.4 at an initial population density (*Pi*) of 4 eggs and $J_2 ml^{-1}$ soil. The obtained results could be used as a base to establish field experiments that allow strategies to prevent surpassing the threshold of nematodes in fields that are infested.

Keywords *Capsicum annuum* · *Meloidogyne* sp. · Nematode reproduction · Threshold level

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Introduction

Pepper (*Capsicum annuum*) is an important horticultural plant in intensive agriculture, particularly in southeast Spain. Pepper suffers various pests and diseases associated with the difficulty of alternating greenhouse crops and, above all, because of the economic consequences of fallow years. Of the diseases that regularly attack pepper plantations, those caused by soil pathogens are the most important because they may make farming non-viable given the difficulty of eradicating endemic diseases. One of these diseases is caused by the cosmopolitan root-knot nematode *Meloidogyne* spp., which is often considered the most damaging genus of plant-parasitic nematode of Mediterranean basin (Sikora

and Fernández 2005) and has a broad host range among Solanaceous plants.

In pepper, a complex system of resistance to root-knot nematodes, involving at least five genes, has been described. Two of these genes (*Me*1 and *Me*3), originally identified in two independent breeding lines, confer the same resistance to the three main species *M. arenaria*, *M. incognita* and *M. javanica*. The three other genes (*Me*2, *Me*4 and *Me*5) induce a more restricted resistance response, being only active against either one *Meloidogyne* nematode species or some populations of species (Castagnone-Sereno et al. 2001).

Knowledge of the initial nematode population density in the soil at sowing is crucial for designing effective environmental friendly control measures (Sasanelli 1994). It is well known that the extent of crop growth impairment by nematodes is influenced by the nematode population density at transplanting, and that a minimum population density (*T*) is required before measurable yield losses occur. This is defined as the tolerance limit (Seinhorst 1965). Although there are some studies based on pepper-nematode interactions (Di Vito 1985; Di Vito et al. 1985, 1992), there is no similarity in the data provided for the threshold level and host-parasite relationships of the peppers tested. It is well known that tolerance limits are directly related to the variety of pepper and nematode, as well as environmental conditions and culture, those limits would establish if a soil is suitable or not for growing pepper.

The present study was carried out on pepper plants infected (around 80 eggs + second stage juvenile (J_2) ml^{-1}) in a commercial field at Campo de Cartagena (southeast Spain) to determine: (1) the nematode species in question; (2) the relationship between the initial population density of the nematode and the growth of pepper seedlings; and (3) the histopathology of nematode-feeding sites in pepper roots infected by *M. incognita*.

Materials and methods

Nematode diagnosis

Samples of pepper roots severely infected by root-knot nematodes were collected from a commercial field in Campo de Cartagena, southeast Spain, during September 2009. For diagnosis and identification, females were collected directly from galled roots. To obtain females for microscopy and molecular analyses, the nematode population was increased on pepper (*Capsicum annuum* cv. California Wonder) in a greenhouse at 25±3°C. Sixty days after inoculation, pepper plants were uprooted, and then washed. The root tissues were teased apart with forceps and transfer needles to remove adult females.

For microscopic examinations, females were mounted in glycerin. Twenty five adult females from this population were analyzed by perineal pattern morphology and excretory pore position. Perineal patterns and anterior body portions were prepared as described by Hartman and Sasser (1985) and examined under a light microscope.

For the differential host test, the response of the *M. incognita* population to different hosts was tested in a greenhouse at 25±3°C, following Vovlas et al. (2008). The differential host set included cotton (*Gossypium hirsutum* cv. Delta Pine 16), peanut (*Arachis hypogaea* cv. Florunner), tobacco (*Nicotiana tabacum* cv. NC 95), watermelon (*Citrullus vulgaris* cv. Charleston Grey) and tomato (cv. Rutgers).

For DNA analysis, single adult females were collected for DNA extraction according to Castillo et al. (2003). For PCR reactions, SCAR primers described by Zijlstra et al. (2000) were used to identify the parasite (Table 1). Amplification reactions were carried out in 25 µl reaction volumes containing 1× buffer, 1.5 mM $MgC1_2$, 0.25 pM each primer, 200 µM each of dATP, dCTP, dGTP and dTTP, 1 U of Taq DNA polymerase (ECOGEN, S.R.L., Spain) and 5 ng of total DNA. The thermocycler was programmed for 2 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at the annealing temperature and 1 min at 72°C. The annealing temperatures were 54°C using the SCARs primers Finc/Rinc, 61°C using the SCARs primers Far/Rar and 64°C using the SCARs primers Fjav/Rjav. DNA primers (5′-TTGATTACGTCCCTGCCCTTT-3′ and 5′-TTTCACTCGCCGTTACTAAGG-3′) spanning the ITS region of most nematodes were used as a control of the amplification quality of each sample (Vrain et al. 1992). PCR products were resolved on 1.5% agarose gels by means of ethidium bromide detection. Gels were documented using a gel documentation and image analyser (Vilber Lourmat, Germany). Reactions were repeated at least twice and always included negative (without DNA) and positive controls with DNA from *M. arenaria, M. javanica* and *M. incognita* adult females.

Table 1 Nucleotide sequence of primer used for Nematode identification

Primer name	Sequence (5′-3′)	Size of the band (bp)	Nematode	Reference
Far Rar	TCGGCGATAGAGGTAAATGAC TCGGCGATAGACACTACAACT	420	*M. arenaria*	Zijlstra et al. 2000
Finc Rinc	CTCTGCCCAATGAGCTGTCC CTCTGCCCTCACATTAAG	1,200	*M. incognita*	Zijlstra et al. 2000
Fjav Rjav	GGTGCGCGATTGAACTGAGC CAGGCCCTTCAGTGGAACTATAC	670	*M. javanica*	Zijlstra et al. 2000

Pair shown was used in PCR reactions using two primers to specifically detect a single species

Inoculum density and plant growth

The stock nematode culture used in this study was derived from infected roots of pepper plants taken from a commercial field in Campo de Cartagena (Murcia) in southeast Spain. The inoculum of *M. incognita* was increased on pepper plants cv. California Wonder as described above. The pepper plants were kept in a greenhouse at 25±3°C. Inoculum was obtained by extracting eggs + J_2 using 1% sodium hypochlorite solution (Hussey and Barker 1973). Three months after inoculation, when egg masses were well formed in the pepper roots, the inoculated plants were uprooted and their roots gently washed free of adhering soil and finely chopped. To estimate the numbers of eggs and J_2 formed in the chopped tissue, six aliquots of 5 g of infected chopped roots were suspended in 1% sodium hypochlorite solution in 100 ml flask for 1 min, and then washed three times with distilled water. Finally, the eggs and J_2 released into suspension were counted (Hussey and Barker 1973). For inoculation, the appropriate amounts of the inoculums were mixed in a potting mixture of autoclaved-sterilized medium (peat-perlite; 1:1) to give a range of increasing initial population (P_i) densities: 0, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 eggs and $J_2 ml^{-1}$ of soil (Di Vito et al. 1986).

Pepper plants were grown from seeds in a greenhouse at 25±3°C in pots containing autoclaved-disinfected medium. When the fifth or sixth true leaf had developed, plants (around 3-month-old) were transplanted and inoculated as described above. The pots were arranged on benches in a randomized block design with six replicates of each population density and one non-infected control. Data on the appearance of symptoms of nematode attack (stunting and yellowing) were recorded during the experiment.

Sixty days after transplanting, the 5-month-old plants were uprooted and the roots were washed free of adhering soil. Plant height and fresh top weight were recorded. Root infection by the nematode was assessed following the root-knot rate chart of Bridge and Page (1980) by estimating root galling severity (RGS) on a 0–10 scale: 0, no knots on root; 1, few small knots but very difficult to find; 2, small knots only but clearly visible, main roots clean; 3, some larger knots visible but main roots clean; 4, larger knots predominate but main roots clean; 5, 50% of roots infested, knotting on some main roots and reduced root system; 6, knotting on main roots; 7, majority of main roots knotted; 8, all main roots, included tap root, knotted and few clean roots visible; 9, all roots severely knotted, plant usually dying; 10, all roots severely knotted, no root system and plant usually dead. Nematode density (eggs and J_2 in the egg masses) in roots was calculated according to Hussey and Barker (1973). Nematodes in soil were extracted as in Di Vito et al. (2004). The final nematode population density (*Pf*) in each pot was determined by summing the nematodes recovered from soil and roots. The reproduction rate (*Pf*/*Pi*) was also calculated for each initial population density.

Statistical analysis

The relationship between the initial nematode population density (*Pi*) and plant growth (estimated from the fresh top weight and plant height) was determined by fitting the data to the Seinhorst model: $y = m + (1-m)\, z^{P-T}$ when $P \geq T$, and $y=1$ when $P < T$ (Seinhorst 1965). In this model, y is the relative value of the plant growth parameter; m is the minimum value of y (y at a very large initial nematode population density); P is the initial nematode population density; T is the

tolerance limit (initial population at which plant growth is not impaired); and z is a constant <1 reflecting nematode damage, with $z^{-T}=1.05$ (Seinhorst, 1965). The coefficient of determination (R^2) and the residual sum of squares were used to indicate the goodness-of-fit of data to the model. T was determined with the least square method.

The greenhouse experiment was performed twice and the similarity between the experiments was tested by preliminary analyses of variance using experimental runs as factors. This allowed the experiment × treatment interaction to be determined; this interaction was not significant ($P\geq0.05$), which allowed data to be combined for analyses of variance and fitting to the Seinhorst model. All data were normalized before analysis by transforming them into $\log_{10}(X+1)$ (Gomez and Gomez 1984). Analyses of variance were carried out using SPSS version 13.0.

Histopathology

Galled roots from 5 month old pepper plants artificially infected as described in the inoculum-density/plant-growth experiment were selected for histopathological studies according to Vovlas et al. (2008).

Results

Pepper cv. California Wonder plants growing in Campo de Cartagena showed severe symptoms, such as stunted growth, yellowing, galls in the root system and low yields, all of which suggested the presence of nematodes. Large, spherical, regular galls were observed in the roots of the affected pepper plants (Fig. 1a). All randomly chosen galls contained egg masses and, in many cases, females were found associated with the bigger galls. Females were probably present in the smaller galls, although to a lower extent. A mean population of 80 eggs and J_2 per ml of soil was estimated.

Nematode diagnosis

Morphological observations showed perineal patterns with high dorsal arch and no lateral lines; head of males with centrally concave labial disc raised above medial lips; anterior portion of male stylet paddle shape, blunt; male stylet knobs rounded to broadly elongate. All these data agree with typical traits of *M. incognita*.

To confirm that the morphometric analysis was correct and that the parasite was *M. incognita*, different hosts were inoculated with the nematode population obtained as described in Material and Methods. The parasite was unable to parasitize cotton, peanut or tobacco, but was able to reproduce on tomato and watermelon, showing that the nematode identification was correct.

As a complementary test, the DNA analysis from natural and artificial infected pepper plants (Fig. 2) confirmed the presence of a nematode because of the intense band of 800 bp (lane 2) amplified with the ITS of most nematodes. When the set of SCAR primers (Table 1) described by Zijlstra et al. (2000) was used to detect the different *Meloidogyne* species, only the combination of primers Finc/Rinc provided an amplified band with the specific size of 1,200 bp (Fig. 2, lane 4) using the DNA extracted, demonstrating that the nematode infecting pepper at that time was *M. incognita*. The DNA control for *M. incognita*, *M. arenaria* and *M. javanica* gave an amplicon of 1,200 bp (Fig. 2, lane 3), 420 bp (Fig. 2, lane 5) and 670 bp (Fig. 2, lane 7), respectively.

Inoculum density and plant growth

Symptoms of *M. incognita* attack on pepper plants (stunting, yellowing and reduction of plant top growth) were evident 40 days after artificial inoculation with an initial population of five eggs and J_2 per ml soil. Fifty days after inoculation, all plants inoculated with more than ten eggs and J_2 per ml of soil showed symptoms and clear and gradual stunting. The root galling severity (Table 2) varied significantly among the initially applied population densities. The maximum nematode reproduction rate (Pf/Pi) was 315.4 at an initial population density (Pi) of four eggs and $J_2 ml^{-1}$ soil (Table 2). This reproduction rate decreased as the initial nematode population increased from 4 to 64 eggs and J_2 per ml of soil. As the initial nematode population density increased, the relationship between fresh top weight and plant height was increasingly well described by the Seinhorst equation (Fig. 3). The tolerance limit (T) for pepper cv. California Wonder plants to *M. incognita* was estimated as 0.85 eggs and $J_2 ml^{-1}$ soil for plant top weight and height (Fig. 3). The minimum relative values (m) for plant height and top fresh weight were 0.15 and 0.16, respectively, at $Pi\geq64$ eggs and $J_2 ml^{-1}$ soil. An initial population density of this pathogen exceeding 32

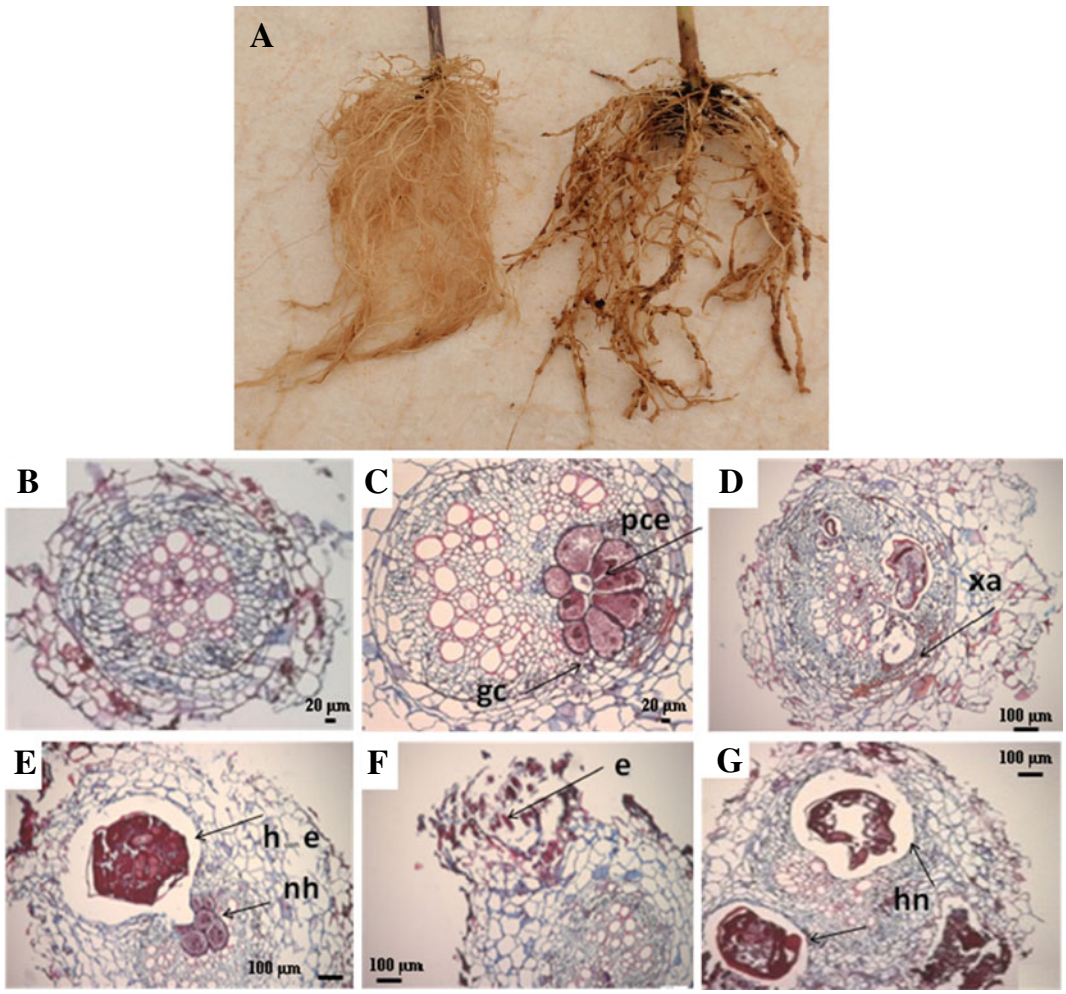

Fig. 1 **a** Severely galled (right) and healthy (left) root system of 5 month old pepper plants infected by *M. incognita*. **b** Cross-sections of healthy and **c–g** root of 5 months old pepper plants infected with nematodes. Abbreviations: gc = giant cells; pce = thickened cell walls; xa = abnormal xylem; he = female with eggs; nh = nucleus hypertrophied; e = eggs; hn = female nematode

eggs and $J_2 ml^{-1}$ soil decreased plant growth by 80% compared with non-infected control plants. The R^2 values of the models were 0.99 for relative top weight and 0.98 for relative plant height (Fig. 3).

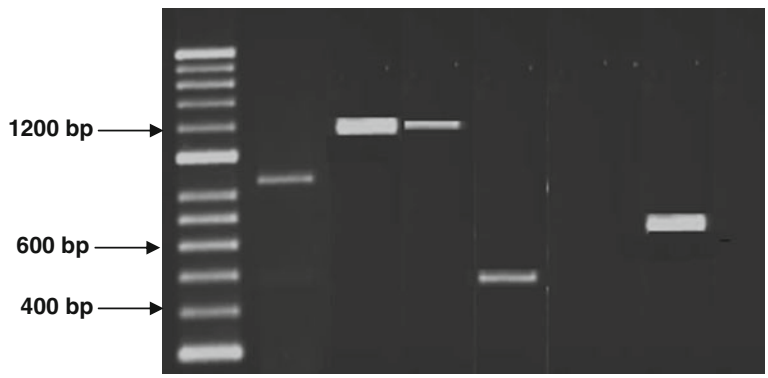

Fig. 2 PCR amplification products using 5 ng template DNA (positive controls: lanes 3, 5, 7; samples: lanes 2, 4, 8) with primers Finc/Rinc (lanes 3, 4), Far/Rar (lanes 5, 6), Fjav/Rjav (lane 7, 8), and IST (lane 2)

Table 2 Relationship between initial population density (P_i) of *Meloidogyne incognita* and root galling severity (RGS), final population density (*Pf*) and reproduction rate (Pf/P_i) on pepper plants, 60 days after inoculation

Initial population density (*Pi*)[a]	RGS	Final population density (*Pf*)	Reproduction rate (*Pf*/*Pi*)
0	0.0 j	0.0 k	0.0 k
0.125	0.0 j	23.1 j	184.8 g
0.250	0.3 i	70.8 i	283.2 b
0.500	1.1 h	120.5 h	241.0 d
1	2.3 g	210.7 g	210.7 f
2	3.2 f	436.2 f	218.1 e
4	5.7 e	1261.8 e	315.4 a
8	6.3 d	2197.5 d	274.7 c
16	7.4 c	2846.3 c	177.9 h
32	8.8 b	3734.1 b	116.7 i
64	9.7 a	4211.8 a	65.8 j

[a] Each pepper seedling was transplanted into a 1,000 ml clay pot with a potting mixture infested with the proper *Pi* (eggs and juveniles per ml soil). Plants were grown in a glasshouse at 25±3°C for 2 months. Data are the averages of two trials, each with six replicated plants per treatment combination

The pepper cv. California Wonder used in this study is shown to be sensitive to *M. incognita*. With a *Pi* of 3 eggs and J_2 per ml soil, plant growth plummets and the soil should not be used to grow peppers if not thoroughly cleansed before the new planting.

Histopathology

Histological preparations were made with healthy (Fig. 1b) and artificially infected (Fig. 1c–g) pepper roots. Substantial modifications in the root architecture were observed. The typical formation of giant cells closely associated with the phloem system is shown in Fig. 1c. Giant cells showed dense cytoplasm and variable numbers of hypertrophied nuclei and nucleoli. Hyperplasia of the tissues adjacent to the giant cells contributed to the formation of root galls. Modifications of the cytoplasm and cortex, endodermis and pericycle were observed (Fig. 1d–e).

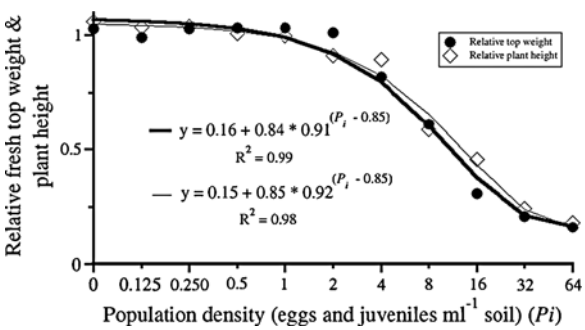

Fig. 3 Relationship between initial densities (*Pi*) of a population of *Meloidogyne incógnita* and relative top fresh weight and relative plant height of pepper cv. California Wonder grown in pots at 25±3°C in a greenhouse for 60 days. Each point represents the average of two trials with six replicated plants each. Line represents the predicted function calculated by fitting the Seinhorst model to data of fresh top weight

Discussion

The starting point of this study was the discovery that some pepper plants in Campo de Cartagena (one of the main pepper production zones in Spain) showed signs of stunting and yellowing followed by low fruit production. It became increasingly clear that if the area was to continue as a productive area the source of the disturbance would have to be identified. The galling observed in the roots of the pepper plants affected (Fig. 1a) suggested that the pathogen was a *Meloidogyne* sp. type nematode, but its formal identification was a prerequisite for the use of appropriate sanitation and disease control methods.

The morphological characterization, the differential host test and the DNA analysis (Fig. 2) showed that the nematode infecting pepper plants was *M. incognita*. Indeed, the results were very clear and these methods, as Zijlstra et al. (2000) suggested, can be

applied for routine diagnostic purposes using DNA extracts from infected plant material. As a result, the set of PCR primers (Table 1) with the morphological characterization and the differential host test can be considered sufficient to differentiate between possible *Meloidogyne* species (*M. incognita, M. arenaria* and *M. javanica*) present in a sample and, consequently increase the possibility of their control.

The histological studies revealed a typical susceptible reaction of the pepper cv. California Wonder plants to infection by *M. incognita* (Fig. 1b–g). The giant cells and tissue changes produced by nematodes sequester the host plant nutrients and limit water and nutrient translocation from infected roots to the upper plant tissues (Agrios 2005).

It is known that damage is usually proportional to the nematode population density, but there are several qualifications which should be made in this respect. The relationship is usually curvilinear, increasing numbers of nematodes having proportionally diminishing effects. In our results, a reduction of the nematode reproduction rate with increasing initial nematode inoculum density was detected (Table 2). These findings could be a consequence of nematode competition for nutrients or root tissue availability (feeding sites), as a result of which the proportion of inoculum developing successfully would diminish.

The nematode, the host and the environment are the three interacting variables influencing the extent of yield loss in infected soils. An understanding of the mechanisms and principles involved in these interacting relationships is essential to predict yield reductions from estimates of pre-planting nematode population densities (*Pi*). So, considering the same root knot nematode (*M. incognita*) in different hosts: in the interaction between *M. incognita* and spinach (Di Vito et al. 2004), the tolerance limit was 0.5 eggs and J_2 per ml of soil and the highest rate of reproduction of the nematode was obtained with *Pi*= 0.0625 eggs and J_2 per ml of soil; in the interaction *M. incognita*—celery (Vovlas et al. 2008) the tolerance limit was 0.15 eggs and J_2 per ml and the maximum reproduction rate was 407.6 from an initial population of 4 eggs and J_2 per ml of soil. In our experiments in the interaction *M. incognita*—pepper, the tolerance limit was 0.85 and *Pi* 4. From the data analyzed concerning the interactions of *M. incognita* with different plants, it may be concluded that there is no a direct relation between initial dose of nematode and the tolerance limit in each plant-nematode interaction.

On the other hand, the results presented by other authors, working in the interaction *M incognita*—pepper, varied. For example, Di Vito et al. (1985) compared the effect of different nematode population densities on sweet pepper production in different crop cycles (1981 and 1983). The tolerance limits obtained were 2.2 and 0.165 eggs and J_2 per ml of soil and a minimum yield of 0.58 and 0.20, respectively, for each crop cycle. The authors suggested that these differences may be due to the different infection techniques used with different initial inoculum levels, even though the soil and environmental conditions were very similar in both experiments. However, Lindsey and Clayshulte (1982) obtained similar values to those reported by Di Vito et al. (1985) in the crop cycle of 1983 but using a different variety of pepper plants (chile peppers).

Data reported by Di Vito (1985), in which he compared a pepper (Yolo Wonder) susceptible to *M. incognita* with a resistant line (line 85558), showed that the tolerance limit was the same for both varieties (T=0.74), while the relative minimum yield varied from 0.1 (susceptible) to 0.4 (resistant). Subsequently, Di Vito et al. (1992) analyzed the same susceptible pepper variety (Yolo Wonder) and a different resistant line (line 89422), obtaining the same tolerance limit (T=0.3) for both varieties but this limit differed from the data obtained by the same author in 1985. Also the percentage of growth suppression (relative minimum yield) was 0.16 for the susceptible variety and 0.5 for the resistant one. These differences are of great note because the same authors obtained different results as regards the tolerance limit, while using the same susceptible variety in both experiments.

Data obtained in this study showed that the tolerance limit of pepper to *M. incognita* was 0.85 eggs and J_2 per ml of soil for the height and the aerial part of the plant. This result differs from those obtained by Di Vito et al. (1992) using sweet peppers but is similar to those obtained by Di Vito (1985). However, minimum relative yields (0.16 for top weight and 0.15 for plant height) for California Wonder variety are similar to those obtained for Yolo Wonder, but different from those obtained for the pepper lines resistant to *M. incognita*. In conclusion, to ascertain the tolerance limit to the pathogen and to evaluate the development of the disease, not only is the type of host plant studied

crucial, but also the growing conditions—soil type, pH, moisture content and temperature. The reproduction rate of nematodes is inversely related to the initial population, although there is a limit of tolerance to the pathogen, which may vary between varieties.

The results obtained in this study, together with those of other authors, suggest that it is desirable to obtain the tolerance limit and the minimum relative yield as a basis for establishing field experiments that will identify strategies (Oka 2010) to prevent surpassing the threshold of nematodes in fields that are infested. In this case, different strategies could be implemented to gradually reduce the level of infestation and keep it below the limit of tolerance defined for pepper growth in the present study.

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