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Resistant accessions of wild *Psidium* spp. to *Meloidogyne enterolobii* and histological characterization of resistance

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Meloidogyne enterolobii has been reported in some states of Brazil and other countries causing severe damage on commercial guava (*Psidium guajava*). The use of resistant varieties is the most effective way to manage nematode parasitism. This study screened 51 accessions of *Psidium* spp. selected from the *Psidium* Germplasm Collection (Embrapa) to look for resistance against *M. enterolobii*. Six months after inoculation, nematode reproduction factor (RF) was used to assess resistance. The following species were resistant to *M. enterolobii*: *P. cattleianum* (yellow guava), *P. friedrichsthalianum* (Costa Rican guava), *Acca sellowiana* (feijoa) and *P. rufum* (purple guava). All 43 wild accessions of *P. guajava* were susceptible, as well as three accessions of *P. guineense* (Brazilian guava), one of *P. acutangulum* (pear guava) and the susceptible control *P. guajava* cv. Paluma. When used as rootstocks under greenhouse conditions, *P. cattleianum* and *P. friedrichsthalianum* were compatible with cv. Paluma; however, in greenhouse and field conditions only 50% of both scions survived. No apparent hypersensitive response (HR) was seen in the resistant guava *P. cattleianum* and *P. friedrichsthalianum*. Juveniles were able to develop normal feeding sites similar to those in susceptible roots 6–13 days after inoculation (dai). From 27 to 32 dai, giant cell deterioration was observed and nematodes showed arrested development. The majority of nematodes failed to reach maturity and did not begin laying eggs in resistant roots. These results suggested that the induction of resistance is relatively late in this pathosystem.

Keywords: genetic resistance, grafting compatibility, histopathology, *Psidium guajava*, wild guava

Introduction

The root-knot nematode (RKN) *Meloidogyne enterolobii* (syn. *M. mayaguensis*) was described from a population sampled in China and isolated from a tree species (*Enterolobium* sp.). This nematode was also reported from other regions in China, mainly isolated from guava (*Psidium guajava*). The presence of this parasite has increasingly been detected worldwide from a wide range of hosts, including crops carrying resistance genes to major *Meloidogyne* spp. (Carneiro *et al.*, 2006; Brito *et al.*, 2007). Recently, *M. enterolobii* was detected in two commercial greenhouses in tomato (*Solanum lycopersicum* cv. Maxifort) resistant to *Meloidogyne* spp. in Switzerland (Kiewnick *et al.*, 2008). Considering the risk of introducing and disseminating this pathogen in the European region, *M. enterolobii* was recently added to the 2012 EPPO alert list as a quarantine nematode.

Morphological identification demands considerable skills and can be unreliable as a result of significant intraspecific morphological variation in *Meloidogyne* spp. Because of its morphological resemblance to *M. incognita* and *M. arenaria* when only the perineal patterns are considered (Carneiro *et al.*, 2001; Brito *et al.*, 2004), *M. enterolobii* has been misidentified in a number of surveys. It is probable that the severe root-knot problem on guava in the Americas and the isolated cases in Africa and Asia involve only this particularly virulent species (Carneiro *et al.*, 2012).

In Brazil, *M. enterolobii* was originally detected on guava orchards in 2001 in Pernambuco and Bahia states (Carneiro *et al.*, 2001). Since then, this nematode has been a matter of major concern in the country because it has spread rapidly, making the cultivation of guava unviable in heavily infested areas (Carneiro *et al.*, 2007; Siqueira *et al.*, 2009).

Typical symptoms of *M. enterolobii* in guava are a strong tanning of leaves and branches, followed by general yellowing of plant aerial parts, culminating in leaf fall and plant death. Infected roots show multiple galls and generalized necrosis, as well as a drastic decrease in fine roots. Moderate infections are associated with common chlorosis, nutrient deficiency and reduced flowers and fruits. *Meloidogyne enterolobii* infection in guava is a complex disease, usually associated with soil fungi infestations (Gomes *et al.*, 2010).

In the São Francisco Valley, a major guava producing region in northeast Brazil, 70% reduction was reported

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in guava production within a 7 year period (2000–2006; Plantec/Codevasf, SGAN 601, Ed. Manoel Novaes, Brasília-DF, Brazil, personal communication). Moreover, as this is an important area for seedling production, *M. enterolobii* may have been spread to other regions throughout Brazil (Carneiro *et al.*, 2007). According to Pereira *et al.* (2009), direct losses associated with guava infection by *M. enterolobii* in several states in Brazil might reach up to US$ 61 million, not to mention direct job losses as a result of decline in guava orchards. Taken together, these observations reveal that new control methods should be undertaken in order to reduce the major economic impact of this *Meloidogyne*-induced disease on guava. Some screenings for resistance against *Meloidogyne* spp. in *Psidium* spp. have been carried out, including one grafting assay; however, in the majority, either the nematode was not identified as *M. enterolobii* (Carneiro *et al.*, 2012) or the rootstocks were misidentified. In a previous study, *P. cattleianum* was resistant (reproduction factor, RF, = 0) and *P. friedrichsthalianum* moderately resistant (RF = 1·9) to *M. enterolobii* (Carneiro *et al.*, 2007).

This work investigates accessions of guava and its wild relatives (*Psidium* spp.) to identify novel sources of resistance to *M. enterolobii*, with the identification of high levels of resistance in *P. cattleianum* and *P. friedrichsthalianum*. Histological observations revealed that the resistance is a late defence response that resulted in the degradation of feeding sites, and production of males, but not adult females or eggs.

Materials and methods

Accessions of *Psidium* spp.

Accessions of *Psidium* spp. used in this study were collected from different regions in Brazil and also from Costa Rica (*P. friedrichsthalianum*). These accessions are deposited in the CENARGEN bank and are specified in Table 1. The commercial *P. guajava* cv. Paluma was used as a susceptible control.

Nematode inoculation

A population of *M. enterolobii* collected in Petrolina (Pernambuco state, Brazil) was used in this study because of its pathogenicity on commercial guava. The identification of species was done using the esterase (Est) phenotype (Carneiro & Almeida, 2001) and SCAR markers as described (Tigano *et al.*, 2010). Prior to inoculation, the population was multiplied in tomato cv. Santa Clara for 3 months under greenhouse conditions. Eggs were extracted from infected roots using 0·5% NaOCl, according to Hussey & Barker (1973), using a blender instead of manual agitation. For histopathological studies, freshly hatched second-stage juveniles (J2) were collected using a modified Baermann funnel. In both cases, counting was done under a light microscope using Peters' slides.

Nematode resistance in controlled conditions

Plants of each accession were grown in 5 L pots filled with a mixture (1:1) of autoclaved soil and Plantimax compost under greenhouse conditions. Seedlings of about 15–20 cm were inoculated with 5000 eggs of *M. enterolobii* by pipetting the nematode suspension around the stem base. Plants were arranged in a completely randomized design with 52 treatments (accessions of wild *Psidium* and guava) and six replications. They were maintained under greenhouse conditions at *c.* 25–30°C and were watered and fertilized as required. Six months after inoculation, the root system was rinsed with tap water and weighed. Roots were stained with phloxine B and evaluated for gall and egg mass numbers (galling index, GI; egg mass index, EMI), using a 0–5 scale, where 0 = no galls or egg masses; 1 = 1–2; 2 = 3–10; 3 = 11–30; 4 = 31–100; and 5 = >100 galls or egg masses per root system (Taylor & Sasser, 1978).

Eggs were extracted using a modified extraction method according to Hussey & Barker (1973), using a blender instead of manual agitation and 1% NaOCl. Total egg number per plant was quantified under a light microscope using Peters' slides. The reproduction factor (RF) was calculated as RF = FP/IP, where FP = final nematode population and IP = initial nematode population (IP = 5000). The average RF was transformed as $\log_{10}(x + 1)$, submitted to analysis of variance and the means separated using the Scott–Knot test at 5% confidence level. The accessions for which RF = 0 were considered immune, RF < 1 resistant (R), and those for which RF ≥ 1 were considered susceptible (S; Sasser *et al.*, 1984).

Plant grafting in greenhouse and field conditions

Twelve-month-old plants of *P. cattleianum*, *P. friedrichsthalianum*, *P. guajava*, *P. rufum* and *A. sellowiana* were grafted with *P. guajava* cv. Paluma using the budding technique. Successful grafted plants (*P. cattleianum*, *P. friedrichsthalianum* and *P. guajava*) were planted in a field infested with *M. enterolobii*. The plants were evaluated in May and September of 2009 and February, June and October of 2010. Plant height and diameter in the grafted region were evaluated.

Histopathological observations

Plantlets of *P. cattleianum* and *P. friedrichsthalianum* accessions and the susceptible control *P. guajava* cv. Paluma were grown in 3 L pots containing washed sterilized sand and were fertilized weekly. Plantlets were inoculated with 20 000 J2 of *M. enterolobii* per plant. Six plantlets of each accession and three for the susceptible control per time point were carefully removed from cups at 3, 6, 12, 16, 18, 22, 28, 34 and 45 days after inoculation (dai), and their roots rinsed with water. Some of the roots from susceptible and resistant plants were stained with acid fuchsin to observe J2 penetration, localization and subsequent development within the roots. After staining, root segments were observed under a stereomicroscope and those parts that showed nematode infection were mounted on a slide for observation under a light microscope (Axiophoto Zeiss).

Subsamples of roots were embedded in resin to produce thin sections. Root fragments showing galls/swellings or without symptoms were excised under a stereomicroscope, fixed, and embedded in Technovit 7100 epoxy resin (Kulzer Friedrichsdorf) as described by Pegard *et al.* (2005) and according to the manufacturer's recommendations. Unstained root sections were mounted on glass slides and fluorescence was observed after UV excitation (UV filter set A2 Zeiss 02; 488002-0000). The same sections were subsequently stained (1 min at 60°C) with 0·5% toluidine blue in 0·1 M sodium phosphate buffer, pH 5·5 and observed using a light microscope.

Table 1 Response of guava accessions (*Psidium guajava*) and *Psidium* spp. to *Meloidogyne enterolobii*, 180 days after inoculation under controlled conditions

Accession[a]	Root fresh weight (g)[b]	GI[c]	EMI[d]	TNE[e] per g of roots	RF[f]	Reaction[g]
1 Guava (wild, red, Brasília, DF)	37·3 b	5·0	4·8	67 012·2 b	499·9 a	S
2 Guava (wild, white, Brasília, DF)	22·2 c	4·3	4·3	36 556·6 c	162·3 c	S
3 Guava (wild, red, Planaltina, DF)	45·8 b	5·0	5·0	70 538·6 b	646·1 a	S
4 Guava (wild, red, Gama, DF)	55·6 b	5·0	4·0	65 459·6 b	727·9 a	S
5 Guava (wild, white, Planaltina, DF)	37·3 b	4·5	4·5	11 569·8 d	86·3 c	S
6 Guava (wild, white, Gama, DF	26·2 c	5·0	4·7	83 511·4 a	437·6 a	S
7 Guava (wild, red, Sobradinho, DF	42·4 b	5·0	5·0	18 480·1 d	156·7 c	S
8 Guava (wild, white, Brasília, DF)	48·7 b	5·0	4·8	39 671·5 bc	386·4 b	S
9 Guava (wild, red, Brasília, DF)	17·4 c	5·0	5·0	18 646·2 cd	64·9 d	S
10 Guava (wild, white, Sobradinho, DF)	15·7 c	4·3	4·3	14 012·7 d	44·0 d	S
11 Guava (wild, red, Sobradinho, DF)	70·8 a	5·0	5·0	63 107·3 b	893·6 a	S
12 Guava (wild, red, Guará, DF)	18·0 c	4·2	4·2	26 913·6 c	96·9 c	S
13 Guava (wild, white, Guará, DF)	43·3 b	4·8	5·0	56 864·2 b	492·4 a	S
14 Guava (wild, white, Brasília, DF)	30·3 b	5·0	4·7	16 076·3 d	97·4 c	S
15 Guava (wild, red, Brasília, DF)	32·0 b	4·3	4·3	95 041·7 a	608·3 a	S
16 Guava (wild, red, Ceilândia, DF)	74·8 a	5·0	5·0	85 448·6 a	639·2 a	S
17 Guava (wild, white, Ceilândia, DF)	24·7 c	5·0	4·5	61 610·4 b	304·4 b	S
18 Guava (wild, red, Núcleo Band, DF)	55·9 b	5·0	5·0	17 101·9 d	191·2 c	S
19 Guava (wild, red, Brasília, DF)	59·9 b	4·8	4·8	34 731·9 c	416·1 b	S
20 Guava (wild, white, Brasília, DF)	87·9 a	4·7	5·0	20 300·8 c	356·9 b	S
21 *P. cattleianum* (yellow guava, Pelotas, RS)	62·5 b	0·0	0·0	0·0 e	0·0 e	R
22 Guava (wild, white, Brasília, DF)	60·1 b	5·0	5·0	19 966·7 c	240·0 b	S
23 Guava (wild, red, Brasília, DF	32·5 b	5·0	5·0	52 635·9 b	342·1 b	S
24 Guava (wild, red, Campo Morão, PR)	30·8 b	5·0	5·0	70 360·7 b	433·4 b	S
25 Guava (wild, red, Londrina, PR)	43·9 b	4·8	4·3	78 906·6 ab	692·8 a	S
26 Guava (wild, white, Piracicaba, SP)	64·7 b	5·0	5·0	25 849·2 c	334·5 b	S
27 Guava (wild, white, Campinas, SP)	50·1 b	5·0	5·0	55 364·8 b	554·8 a	S
28 Guava (wild, white, Limeira, SP)	54·0 b	5·0	5·0	52 716·1 b	569·3 a	S
29 Guava (wild, red, Limeira, SP)	55·8 b	5·0	5·0	51 366·0 b	573·2 a	S
30 Guava (wild white, Botucatu, SP)	100·5 a	4·5	4·3	27 347·7 c	549·7 a	S
31 Guava (wild, yellow, Botucatu, SP)	76·1 a	5·0	5·0	42 476·3 c	646·5 a	S
32 Guava (wild, white, Indaiatuba, SP)	35·6 b	5·0	4·7	56 354·6 b	401·2 b	S
33 Guava (wild, red, Indaiatuba, SP)	94·0 a	5·0	5·0	11 834·5 d	222·5 c	S
34 Guava (wild, red, Jaboticabal, SP)	32·4 b	5·0	5·0	50 220·6 b	325·3 b	S
35 Guava (wild, white, Jaboticabal, SP)	22·3 c	4·2	4·2	66 288·0 b	295·6 b	S
36 Guava (wild, white, Brasília, DF)	12·7 c	5·0	5·0	22 117·2 c	56·2 d	S
37 *Acca sellowiana* (pineapple guava or feijoa, Capão Alto, SC)	32·6 b	0·0	0·0	0·0 e	0·0 e	R
38 *P. guineense* (Brazilian guava, Brasília, DF)	44·2 b	4·3	4·3	39 259·3 c	33·0 d	S
39 *P. guineense* (Brazilian guava, Divinópolis, GO)	61·4 b	4·7	4·7	16 438·6 d	201·9 b	S
40 *P. guineense* (Brazilian guava, Planaltina, DF)	29·7 b	3·8	3·9	4365·8 d	25·9 d	S
41 Guava (wild, white, GO)	35·3 b	5·0	5·0	58 633·9 b	414·0 b	S
42 Guava (wild, red, Divinópolis, GO)	41·3 b	5·0	5·0	114 210·4 a	943·4 a	S
43 *P. friedrichsthalianum* (Costa Rican wild guava, Turrialba, Costa Rica)	24·1 c	1·0	0·0	337·0 e	0·8 e	R
44 Guava (wild, Brasília, DF)	41·6 b	2·9	2·9	2750·4 d	22·9 d	S
45 Guava (wild, white, Brasília, DF)	96·3 a	5·0	5·0	19 044·7 c	366·8 b	S
46 Guava (wild, white, Brasília, DF)	119·3 a	5·0	5·0	20 106·2 c	479·7 b	S
47 Guava (wild, white, Brasília, DF)	50·5 b	5·0	5·0	25 656·8 c	259·1 b	S
48 Guava (wild, red, Cristalina, GO)	58·8 b	4·1	4·9	15 493·2 d	182·2 b	S
49 Guava (wild, white, Cristalina, GO)	43·3 b	4·9	4·9	22 525·0 c	195·1 b	S
50 *P. rufum* (purple guava, Paracatu, MG)	13·5 c	0·0	0·0	0·0 de	0·0 e	R

(continued)

Table 1 (continued)

Accession[a]	Root fresh weight (g)[b]	GI[c]	EMI[d]	TNE[e] per g of roots	RF[f]	Reaction[g]
51 *P. acutangulum* (pear guava, Belém, PA)	183·7 a	5·0	5·0	9656·4 d	177·4 c	S
52 Control: guava cv. Paluma (Petrolina, PE)	44·3 b	5·0	5·0	19 633·8 c	174·0 c	S

Data were transformed as log ($x + 1$). Means followed by different letters are significantly different according to Scott–Knot's test ($P < 0·05$). Coefficient of variation (CV) = 37%.

[a]Brazilian states: DF, Distrito Federal; RS, Rio Grande do Sul; PR, Paraná; SP, São Paulo; SC, Santa Catarina; GO, Goiás; MG, Minas Gerais; PA, Pará; PE, Pernambuco.

[b]Mean values ($n = 6$) of fresh weight of roots (g).

[c]Mean values ($n = 6$) of gall index (GI) based on a 0–5 scale. 0: no galls, 1: 1–2 galls, 2: 3–10 galls, 3: 11–30 galls, 4: 31–100 galls, 5: >100 galls per root system (Taylor & Sasser, 1978).

[d]Mean values ($n = 6$) of egg mass index (EMI) based on a 0–5 scale. 0: no galls, 1: 1–2 galls, 2: 3–10 galls, 3: 11–30 galls, 4: 31–100 galls, 5: >100 egg masses per root system (Taylor & Sasser, 1978).

[e]Mean values ($n = 6$) of total number of eggs (TNE) per gram of roots.

[f]Mean values ($n = 6$) of reproduction factor (RF = final population/5000 eggs of *M. enterolobii*).

[g]Reaction of inoculated plants. RF ≥ 1 = susceptible, S; RF < 1 = resistant, R.

Results

Characterization of nematode resistance in controlled conditions

The RF values from the species *P. cattleianum*, *P. friedrichsthalianum*, *P. rufum* and *A. sellowiana* indicated that they were resistant (RF < 1) to *M. enterolobii* (Table 1). All wild accessions of *P. guajava* as well as the commercial guava cv. Paluma, *P. acutangulum* and *P. guineense* were susceptible (RF > 1; Table 1). Some wild guava (accessions 9, 10, 36 and 44) showed significantly smaller RF than other *P. guajava*, and similar to accessions 38 and 40 of *P. guineense*.

Evaluation of plant grafting in greenhouse and field conditions

All 10 plants of *P. friedrichsthalianum* (Costa Rican guava) and nine out of 10 plants of *P. cattleianum* (yellow guava) were compatible with guava cv. Paluma, using the budding technique, in greenhouse conditions. None of the 10 grafted plants of *P. rufum* or *A. sellowiana* were compatible.

All successfully grafted plants were planted in the field in May 2009, in an area naturally infested with *M. enterolobii* (Luziânia, GO, Brazil), for evaluation of their development. The evaluations of plant height were done in May and September 2009, and in February, June and October 2010.

Based on data collected up to September 2009, it was observed that *c.* 50% of guava trees grafted on *P. friedrichsthalianum* and *P. cattleianum* died under field conditions. The guava trees grafted on *P. friedrichsthalianum* were vigorous, producing buds, flowers and fruits from June 2010. Plants grafted on *P. cattleianum* showed an incompatibility reaction in the field (enlargement in the grafted region) observed in September 2009. Consequently, all nine plants planted in the field had died by June 2010 (Table 2). Control plants (guava grafted on guava) were taller than plants grafted on *P. friedrichsthalianum* (Table 2). Unfortunately, during the rainy season of October 2010, cattle gained access into the experimental plot and grazed guava shoots, reducing their height (Table 2).

Histopathological observations of *Psidium* spp. inoculated with *M. enterolobii*

The susceptible *P. guajava* cv. Paluma was chosen for the histopathological observations of the compatible reaction (Fig. 1). In the susceptible host, examination of acid fuchsin-stained guava roots and toluidine blue-stained sections showed that J2 were able to penetrate the root tips, migrate along with the sieve elements, and develop normally after having initiated the differentiation of feeding sites (Fig. 1a–c). At 3 dai, numerous J2 were observed localized in the subapical meristem of the roots (Fig. 1a), and J2 were present in the root cortex, probably migrating towards the vascular cylinder; at this stage, cell wall damage (Fig. 1b) was frequently seen. At 6–10 dai, numerous juveniles were observed within the vascular cylinder, and feeding juveniles were visible inside the vascular cylinder close to giant cells (Fig. 1c). At 17–23 dai, numerous J3/J4 had established feeding sites with 6–14 well-formed giant cells containing numerous nuclei, dense cytoplasm, and small vacuoles (Fig. 1d,e). At this time, enlargement of the vascular cylinder region took place, and large galls were apparent. Giant cells displayed thickened cell walls (Fig. 1d–f). At 27 dai, adult females were observed (Fig. 1f) associated with severe injuries to the surrounding cells, disruption of the root cortex and accumulation of egg masses at the root surface (Fig. 1g).

The resistant accessions of *P. cattleianum* and *P. friedrichsthalianum* were used to histologically analyse defence responses of the plant. Because results were very

Table 2 Comparison of grafted plant heights (cm) under field conditions

	Time of year				
Psidium species[a]	May 2009	Sept 2009	Feb 2010	Jun 2010	Oct 2010
P. friedrichsthalianum	102·5 ± 11·3[b]	108·5 ± 12·9	116·7 ± 13·6	118·8 ± 14·2	82·7 ± 4·2[c]
P. cattleianum	97·2 ± 6·20	98·0 ± 6·6	112·5 ± 8·5	–[d]	–[d]
P. guajava	110·0 ± 1·15	111·0 ± 1·2	113·2 ± 1·5	158·8 ± 1·2	122·3 ± 2·2[c]

[a]Rootstocks were grafted with *P. guajava* cv. Paluma. Plant height was measured 90 days after planting in the field.
[b]Mean (n = 9–10) of plant heights (cm) ± SE.
[c]Plant height was affected by cattle grazing.
[d]Scions died.

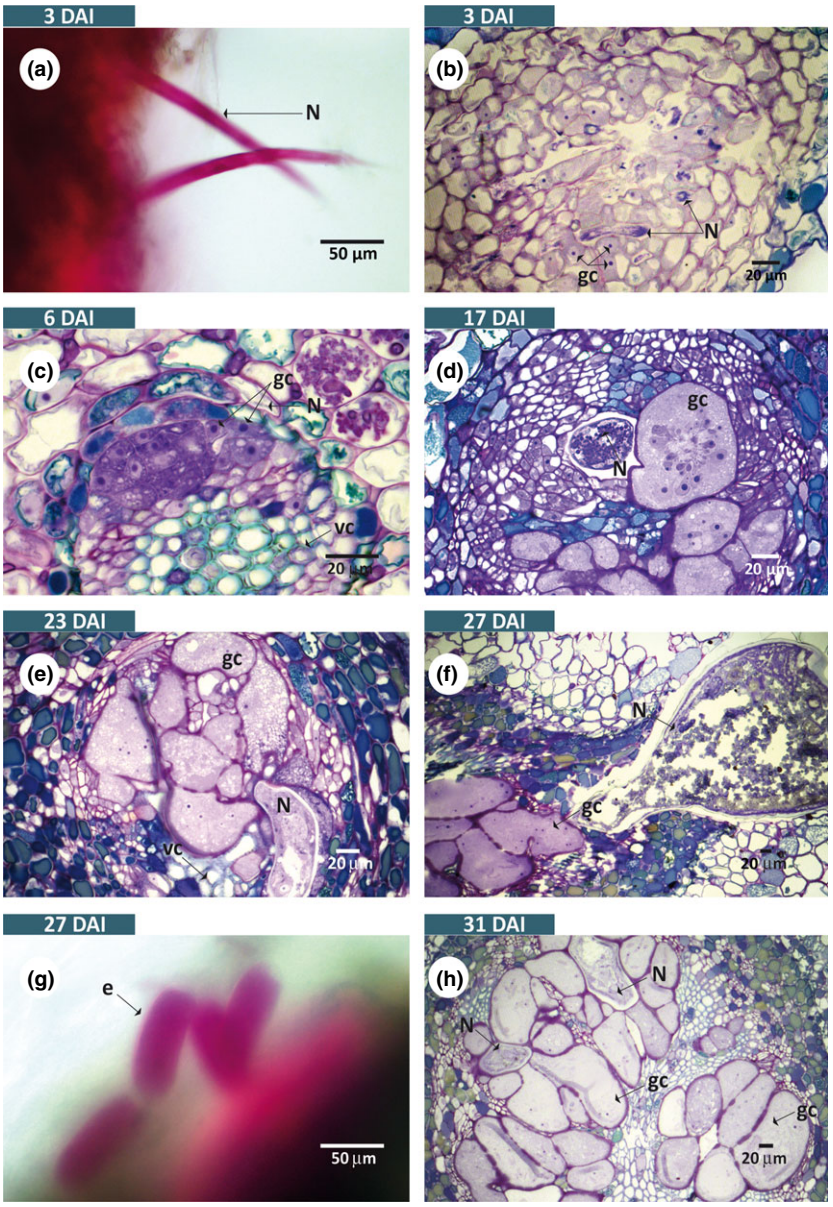

Figure 1 Root sections of *Psidium guajava* cv. Paluma (susceptible) infested with *Meloidogyne enterolobii*. (a) Roots showing J2 at the penetration stage; (b, c) giant cells indicating the differentiation of feeding sites close to the nematode; (d, e) giant cells in different stages of formation along with J3 and J4; (f) an adult female close to giant cells; (g) red-stained eggs; (h) large number of completely formed giant cells. vc, vascular cylinder; gc, giant cells; N, nematodes; E, eggs. Sections visualized after staining with toluidine blue (b, c, d, e, f, h) and acid fuchsin (a, g).

similar, only *P. cattleianum* is shown (Fig. 2). In both accessions, nematode penetration did not seem to be affected, and a lot of J2s could penetrate the resistant guava accessions, as in the susceptible one (data not shown). Microscopic examination of acid fuchsin-stained roots and observations of toluidine blue-stained sections

showed that J2 were localized in the epidermis and cortex at 3–6 dai, indicating that penetration and colonization were in progress (Fig. 2a–c).

The two resistant *Psidium* spp. did not show any HR response to nematode penetration at the early infection stage (Fig. 2b). When roots were examined at 13–17 dai, nematodes were seen close to feeding sites in which the giant cells displayed similar features to those in susceptible roots (Fig. 2d). The first evident differences between the guava cv. Paluma and the resistant *Psidium* spp. were detected at 15–23 dai, when the giant cells adjacent to the nematode in resistant roots had some larger vacuoles that took up almost the entire volume of the cells, whereas the giant cells in the susceptible roots had uniformly dense cytoplasm with less vacuolation (Fig. 2e–h). Prior to 13 dai, some nematodes were developing normally in the resistant accessions based on observations of their size, shape and appearance of internal contents. However, nematodes associated with resistant roots at 22–27 dai were arrested in their development (J4; Fig. 2f) in parallel with the deterioration of giant cells. At this stage, most of the giant cells appeared to be on the verge of collapse and devoid of any visible cytoplasmic content (Fig. 2e), whereas in susceptible roots, robust giant cell complexes with dense cytoplasm and thick cell walls were present (Fig. 1f,h). At 22–31 dai (Fig. 2e,g,h) several males were observed inside the J4 and none were observed to have reached to a mature female stage. The presence of putatively phenolic compounds (blue fluorescent material) was visible inside the body of J4 females and males (Fig. 2h).

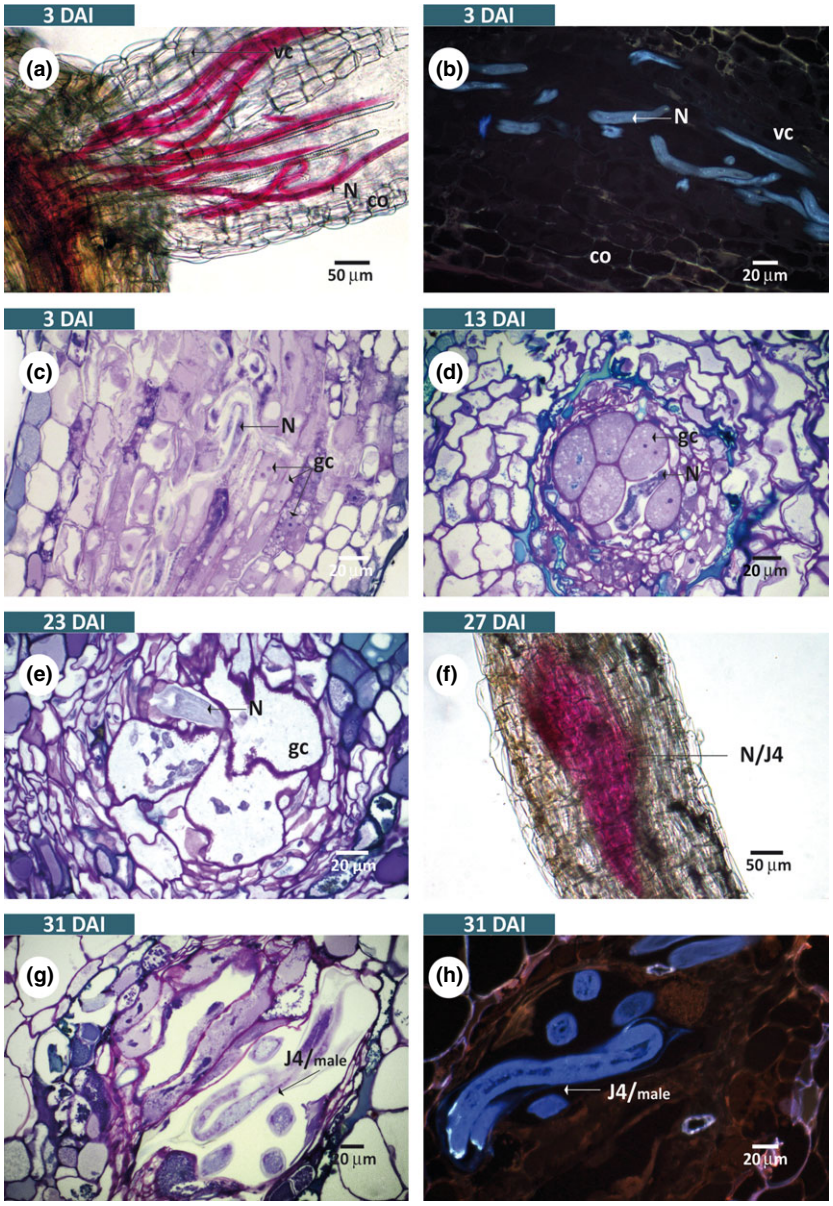

Figure 2 Root sections of *Psidium cattleianum* (resistant) infested with *Meloidogyne enterolobii*. (a) Numerous J2 nematodes at the penetration stage; (b) numerous blue fluorescent J2 nematodes in the vascular cylinder; (c,d) giant cells organized around J3/J4; (e) giant cells with degraded cytoplasm; (f) J4 with arrested development; (g, h) males inside J4, and male with fluorescent blue staining. vc, vascular cylinder; co, cortex; gc, giant cells; N, nematodes. Sections visualized under UV light (b, h); sections stained with toluidine blue (c, d, e, g) and acid fuchsin (a, f).

Discussion

Considering the difficulty in identifying *M. enterolobii* by just the perineal pattern (Carneiro *et al.*, 2001; Brito *et al.*, 2004), it is possible that *M. enterolobii* from guava has been misidentified in several countries, because the identification of root-knot nematodes in this plant species has been based only on morphology and differential host data (Villota & Agudelo, 1997; Crozzoli & Casassa, 1998). Using isozyme and RAPD markers for *Meloidogyne* spp. identification, only *M. enterolobii* was detected in guava in Brazil (Siqueira *et al.*, 2009). Carneiro *et al.* (2012) found that *P. guajava* was a good host for *M. enterolobii* and a non-host for *M. arenaria*, *M. incognita* and *M. javanica*. Rossi *et al.* (2002) observed similar results when three commercial guava cultivars were inoculated with *M. incognita* race 2 and *M. javanica*.

Susceptibility of *P. guajava* to *M. enterolobii* observed in this study was also reported by other authors for *Meloidogyne* spp. (Villota & Agudelo, 1997; Lee *et al.*, 1998; Maranhão *et al.*, 2001; Carneiro *et al.*, 2007; Almeida *et al.*, 2009; Gonzalez-Gaona *et al.*, 2010; Castro *et al.*, 2012). Casassa *et al.* (1997) evaluated seven *P. guajava* accessions 6 months after inoculation with 5000 eggs of *M. incognita*. Resistance was observed in *P. guajava* accession S3 (RF = 0·2). In this study, only the accession 44 of wild guava (Olhos d'água Park) showed good results (GI = EMI = 2·9 and RF = 22·9) when compared with other evaluated wild *P. guajava*. However, more studies under field conditions are necessary to confirm these results. Milan (2007, 2010) also detected resistance in guava accessions from Malaysia. In the first study, *P. guajava* B-12 was classified as resistant to *M. incognita* (RF = 0·88) and in the second report this author verified that three accessions of *P. guajava* (K-10, A-06 and J-16) were resistant to *M. incognita* (GI < 2); however, *M. enterolobii* may have been the species challenged.

Another important aspect is the difficulty of identifying species of *Psidium*. In some studies, *P. friedrichsthalianum* was identified incorrectly as 'Brazilian guava', although it is not native to Brazil. The Brazilian guava was identified as *P. guineense* (C. Proença, Universidade de Brasília, Brazil, personal communication).

In the present study, *P. cattleianum*, *P. friedrichsthalianum*, *P. rufum* and *A. sellowiana* were resistant to *M. enterolobii*. Previous analysis using *Psidium* spp. accessions confirmed the resistance of *P. cattleianum* and *P. friedrichsthalianum* (Cuadra & Quincosa, 1982; Casassa *et al.*, 1997, 1998; Villota & Agudelo, 1997; Maranhão *et al.*, 2003; Carneiro *et al.*, 2007; Milan, 2007; Bogantes-Arias & Mora-Newcomer, 2010). Two accessions of *P. guineense* were classified as susceptible to *M. enterolobii*, but having low RF (25·9 and 33) when compared to that of the control guava cv. Paluma (RF = 174) and accession 39 of *P. guineense* (RF = 201·9). Variation in susceptibility of *P. guineense* was seen following inoculation with *M. enterolobii* (Maranhão *et al.*, 2003; Costa *et al.*, 2012). These accessions were classified from moderately resistant to highly susceptible (RF = 4·76–38·89) and resistant to susceptible (RF = 0–3·47), respectively. The unique *P. guineense* accession tested was evaluated as immune (Lee *et al.*, 1998).

The results of successful grafting are in accordance with those obtained by other authors (Villavicencio *et al.*, 1995; Marin *et al.*, 2000; Bogantes-Arias & Mora-Newcomer, 2010). Vegetative growth was significantly higher in the guava rootstock treatment when compared with *P. friedrichsthalianum* rootstock. A similar observation was made by Bogantes-Arias & Mora-Newcomer (2010) who reported that in the field, guava cv. Paluma grafted on guava rootstock showed more vigorous growth than guava grafted on Costa Rican rootstock, which clearly showed a strong dwarfing effect, whereas Brazilian guava rootstocks (probably *P. guineense*) exhibited an intermediate effect on vegetative vigour. Number and weight of fruits was higher when cultivated guava was used as a rootstock compared to wild guava rootstocks; no differences were observed between two wild guava rootstocks (Bogantes-Arias & Mora-Newcomer, 2010). Recently, interspecific hybrids between *P. guajava* × *P. guineense* selected for resistance to *M. enterolobii* showed high compatibility with guava cv. Paluma, indicating that this strategy may be relevant in controlling *M. enterolobii* (Costa *et al.*, 2012).

Host resistance mechanisms act to prevent *Meloidogyne* development in different plants. The hypersensitive response (HR) is the most common, manifesting either as a pre- or post-infection event, associated with a rapid host cell death surrounding initial infection sites by the pathogen. As a result, the pathogen is arrested and its development is partially or completely inhibited (Williamson & Hussey, 1996; Williamson & Kumar, 2006). Hypersensitive-like reactions have been observed in *Mi*-mediated resistance in tomato (Williamson & Hussey, 1996), *Mex-1* in coffee (Anthony *et al.*, 2005), *Me7* in pepper (Pegard *et al.*, 2005) and on wild peanut–*M. arenaria* (Proite *et al.*, 2008), *Vitis* spp.–*M. arenaria* (Anwar & McKenry, 2002) and coffee–*M. incognita* (Albuquerque *et al.*, 2010) interactions.

In the resistant accessions *P. cattleianum* and *P. friedrichsthalianum*, root penetration by the nematode was not affected, and similar numbers of J2s could be observed in the susceptible guava cv. Paluma and resistant accessions. Similar observations were reported for the moderately resistant cotton cv. Clevewilt accession (McClure *et al.*, 1974), in the highly resistant accession M-315 RNR (Jenkins *et al.*, 1995), and in a number of other resistant accessions (Faske & Starr, 2009).

Interestingly, in the current study there was no evidence of early HR in *P. cattleianum* and *P. friedrichsthalianum*. In fact, the nematode was able to initiate and maintain apparently healthy giant cells in resistant roots for about 2 weeks before visible signs of deterioration occurred, leading to giant cell collapse. A similar mecha-

nism was observed in cowpea roots containing the *Rk* gene (Das *et al.*, 2008). Other authors also reported a delayed resistance against RKN in tobacco (Powell, 1962) and in pepper HDA330 (Bleve-Zacheo *et al.*, 1998), in which a late HR was seen in developed giant cells. In the present study, no HR-like phenotype was observed in the resistant guava, even during the late stage of infection. During this time, nematodes were able to feed and develop normally until reaching the J4 stage. Infections associated with normal feeding site development, without any HR, in resistant plants have also been reported in nematode interactions with cotton, *Cucumis* and cowpea (McClure *et al.*, 1974; Walters *et al.*, 2006; Das *et al.*, 2008).

Male sex conversion when juveniles cannot establish appropriate feeding sites is common when nutritional and environmental conditions are not favourable to nematode development (Fassuliotis, 1970; Williamson & Hussey, 1996; Pofu & Mashela, 2011). In this study, the presence of males can be explained by the fact that alteration of giant cells strongly reduced their metabolic activity, providing suboptimal nutrition for female nematode development up to 28 dai. No males were observed in the proximity of roots or in the soil, indicating that they died inside the roots.

This study contributes to the provision of a strong foundation for the development of resistant guava rootstocks in Brazil and other regions, to be used in the control of *M. enterolobii* under field conditions. The microscopic observations suggest that resistance of wild *Psidium* accessions results from late defence responses with no apparent HR, but severe alteration of feeding sites associated with a lack of female development and egg production.

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References

Albuquerque EVS, Carneiro RMDG, Costa PM *et al.*, 2010. Resistance to *Meloidogyne incognita* expresses a hypersensitive-like response in *Coffea arabica*. *European Journal of Plant Pathology* **127**, 365–73.

Almeida EJ, Santos JM, Martins ABG, 2009. Resistance of guava and araca to *Meloidogyne mayaguensis*. *Pesquisa Agropecuaria Brasileira* **44**, 421–3.

Anthony F, Topart P, Martinez A, Silva M, Nicole M, Silva AR, 2005. Hypersensitive-like reaction conferred by the *Mex-1* resistance gene against *Meloidogyne exigua* in coffee. *Plant Pathology* **54**, 476–82.

Anwar SA, McKenry MV, 2002. Developmental response of a resistance-breaking population of *Meloidogyne arenaria* on *Vitis* spp. *Journal of Nematology* **34**, 28–33.

Bleve-Zacheo T, Bongiovanni M, Melillo MT, Castagnone-Sereno P, 1998. The pepper resistance genes *Me1* and *Me3* induce differential penetration rates and temporal sequences of root cell ultrastructural changes upon nematode infection. *Plant Science* **133**, 79–90.

Bogantes-Arias A, Mora-Newcomer E, 2010. Evaluation of four rootstocks for graft in guava (*Psidium guajava* L.). *Agronomia Mesoamericana* **21**, 103–11.

Brito JA, Powers TO, Mullin PG, Inserra RN, Dickson DW, 2004. Morphological and molecular characterization of *Meloidogyne mayaguensis* from Florida. *Journal of Nematology* **36**, 232–40.

Brito JA, Stanley JD, Mendes ML, Cetintas R, Dickson DW, 2007. Host status of selected cultivated plants to *Meloidogyne mayaguensis* in Florida. *Nematropica* **37**, 65–71.

Carneiro RMDG, Almeida MRA, 2001. Técnica de eletroforese usada no estudo de enzimas dos nematoides de galhas para identificação de espécies. *Nematologia Brasileira* **25**, 35–44.

Carneiro RMDG, Moreira W, Almeida MRA, Gomes ACMM, 2001. Primeiro registro de *Meloidogyne mayaguensis* em goiabeira no Brasil. *Nematologia Brasileira* **25**, 223–8.

Carneiro RMDG, Almeida MRA, Braga RS, Almeida CA, Gioria R, 2006. First record of *Meloidogyne mayaguensis* parasitising resistant root-knot nematode pepper and tomato plants in São Paulo State, Brazil. *Nematologia Brasileira* **30**, 81–6.

Carneiro RMDG, Cirotto PA, Silva DB, Carneiro RG, 2007. Resistance to *Meloidogyne mayaguensis* in *Psidium* spp. accessions and their grafting compatibility with *P. guajava* cv. Paluma. *Fitopatologia Brasileira* **32**, 281–4.

Carneiro RMDG, Freitas VM, Mattos JK, Castro JMC, Gomes CB, Carneiro RM, 2012. Major guava nematodes and control prospects using resistance on *Psidium* spp. and non-host crops. *Acta Horticulturae* **959**, 41–9.

Casassa AM, Matheus J, Crozzoli R, Bravo V, Gonzalez C, 1997. Response of some selections of guava to *Meloidogyne incognita*, in Mara County, Zulia State, Venezuela. *Fitopatologia Venezolana* **10**, 5–8.

Casassa AM, Crozzoli R, Matheus J, Bravo V, Marin M, 1998. Effect of the root-knot nematode, *Meloidogyne incognita*, on the growth of guava (*Psidium* spp.) in nurseries. *Nematologia Mediterranea* **26**, 237–42.

Castro JMC, Santos CAF, Flori JE, Siqueira SVC, Novaes PAR, Lima RG, 2012. Reaction of *Psidium* accessions to the *Meloidogyne enterolobii* root-knot nematode. *Acta Horticulturae* **959**, 51–7.

Costa SR, Santos CAF, Castro JMC, 2012. Assessing *Psidium guajava* × *P. guineense* hybrids tolerance to *Meloidogyne enterolobii*. *Acta Horticulturae* **959**, 59–66.

Crozzoli R, Casassa AM, 1998. Species and strains of *Meloidogyne* in guava at Venezuela. *Revista de la Facultad de Agronomia* **15**, 107–8.

Cuadra R, Quincosa A, 1982. Comportamiento de diferentes especies de *Psidium* como patrones para guayabos resistentes a *Meloidogyne*. *Ciencias de la Agricultura* **13**, 19–26.

Das S, DeMason DA, Ehlers JD, Close TJ, Roberts PA, 2008. Histological characterization of root-knot nematode resistance in cowpea and its relation to reactive oxygen species modulation. *Journal of Experimental Botany* **59**, 1305–13.

Faske TR, Starr JL, 2009. Mechanism of resistance to *Meloidogyne incognita* in resistant cotton genotypes. *Nematropica* **39**, 281–8.

Fassuliotis G, 1970. Resistance of *Cucumis* spp. to the root-knot nematode, *Meloidogyne incognita acrita*. *Journal of Nematology* **2**, 174–8.

Gomes VM, Souza RM, Dias VM, Silveira SF, Dolinski C, 2010. Guava decline: a complex disease involving *Meloidogyne mayaguensis* and *Fusarium solani*. *Journal of Phytopathology* **158**, 1–6.

Gonzalez-Gaona E, Padilla-Ramirez JS, Lozano-Gutierrez J *et al.*, 2010. Evaluation of Mexican guava germplasm against root knot nematodes. *Acta Horticulturae* **849**, 363–8.

Hussey RS, Barker KR, 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. *Plant Disease Reporter* **57**, 1025–8.

Jenkins JN, Creech RG, Tang B, Lawrence G, McCarty JC, 1995. Cotton resistance to root-knot nematode: II post-penetration development. *Crop Science* **35**, 369–73.

Kiewnick S, Karssen G, Brito JA, Oggenfuss M, Frey JE, 2008. First report of root-knot nematode *Meloidogyne enterolobii* on tomato and cucumber in Switzerland. *Plant Disease* **92**, 1370.

Lee MD, Chen CH, Tsay TT, Lin YY, 1998. Survey and control of guava nematode diseases. *Plant Protection Bulletin (Taipei)* **40**, 265–76.

Maranhão SRVL, Moura RM, Pedrosa EMR, 2001. Reação de indivíduos segregantes de goiabeira a *Meloidogyne incognita* 1 e *M. mayaguensis*. *Nematologia Brasileira* **25**, 191–5.

Maranhão SRVL, Moura RM, Pedrosa EMR, 2003. Reação de indivíduos segregantes de araçazeiro a *Meloidogyne incognita* raça 1, *M. javanica* e *M. mayaguensis*. *Nematologia Brasileira* **27**, 173–8.

Marin M, Casassa A, Rincon A *et al.*, 2000. Performance of cultivars of guava (*Psidium guajava* L.) grafted on *Psidium friedrichsthalianum* Berg-Niedenzu. *Revista de la Facultad de Agronomia* **17**, 384–92.

McClure MA, Ellis KC, Nigh EL, 1974. Post-infection development and histopathology of *Meloidogyne incognita* in resistant cotton. *Journal of Nematology* **6**, 21–6.

Milan AR, 2007. Breeding of *Psidium* species for root knot nematode resistance in Malaysia. *Acta Horticulturae* **735**, 61–9.

Milan AR, 2010. Collection and evaluation of guava (*Psidium guajava* L.) for nematode resistance in Malaysia. *Acta Horticulturae* **849**, 357–62.

Pegard A, Brizzard G, Fazari A, Soucaze O, Abad P, Djian-Caporalino C, 2005. Histological characterization of resistance to different root-knot nematode species related to phenolics accumulation in *Capsicum annuum*. *Phytopathology* **95**, 158–65.

Pereira FOM, Souza RM, Souza PM, Dolinski C, Santos GK, 2009. Estimativa do impacto econômico e social direto de *Meloidogyne mayaguensis* na cultura da goiaba no Brasil. *Nematologia Brasileira* **33**, 176–81.

Pofu KM, Mashela PW, 2011. Using relative penetration and maleness indices in *Meloidogyne incognita* to establish resistance type in *Cucumis myriocarpus*. *African Journal of Biotechnology* **10**, 390–3.

Powell NT, 1962. Histological bases of resistance to root-knot nematodes in flue-cured tobacco. *Phytopathology* **52**, 25.

Proite K, Carneiro R, Falcao R, Gomes A, Leal-Bertioli S, Guimaraes P, 2008. Post-infection development and histopathology of *Meloidogyne arenaria* race 1 on *Arachis* spp. *Plant Pathology* **57**, 974–80.

Rossi CE, Ferraz LCCB, Montaldi PT, 2002. Resistance of fruit tree rootstocks to *Meloidogyne incognita* race 2 and *M. javanica*. *Arquivos do Instituto Biológico* **69**, 43–9.

Sasser JN, Carter CC, Hartman KM, 1984. *Standardization of Host Suitability Studies and Reporting of Resistance to Root-Knot Nematodes*. Raleigh, NC, USA: North Carolina State University Graphics.

Siqueira KMS, Freitas VM, Almeida MRA *et al.*, 2009. Detecção de *Meloidogyne mayaguensis* em goiabeira e mamoeiro no estado de Goiás, usando marcadores moleculares. *Tropical Plant Pathology* **34**, 256–60.

Taylor AL, Sasser JN, 1978. *Biology, Identification and Control of Root-Knot Nematodes (Meloidogyne spp.)*. Raleigh, NC, USA: North Carolina State University, US Agency International Division.

Tigano M, Siqueira KMS, Castagnone-Sereno P *et al.*, 2010. Genetic diversity of the root-knot nematode *Meloidogyne enterolobii* and development of a SCAR marker for this guava-damaging species. *Plant Pathology* **59**, 1054–61.

Villavicencio LR, Marín ML, Rincón CC, 1995. Crecimiento de la guayaba (*Psidium guajava* L.) injertada sobre (*Psidium friedrichsthalianum*). *Revista de la Facultad de Agronomia* **12**, 451–65.

Villota FJ, Agudelo FV, 1997. Evaluation of guava material (*Psidium guajava* L.) for the damage behaviour of *Meloidogyne incognita*. *Fitopatologia Colombiana* **21**, 31–8.

Walters SA, Wehner TC, Daykin ME, Barker KR, 2006. Penetration rates of root-knot nematodes into *Cucumis sativus* and *C. metuliferus* roots and subsequent histological changes. *Nematropica* **36**, 231–42.

Williamson VM, Hussey RS, 1996. Nematode pathogenesis and resistance in plants. *The Plant Cell* **8**, 1735–45.

Williamson VM, Kumar A, 2006. Nematode resistance in plants: the battle underground. *Trends in Genetics* **22**, 396–403.