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Post-infection development and histopathology of *Meloidogyne arenaria* race 1 on *Arachis* spp.

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The reproductive behaviour of the root-knot nematode *Meloidogyne arenaria* race 1 was compared on two wild species of *Arachis* (*A. duranensis* and *A. stenosperma*) and cultivated peanut (*A. hypogaea* cv. IAC-Tatu-ST). The three species were considered moderately susceptible, resistant, and susceptible, respectively. Penetration and development of the root-knot nematode in the resistant species was reduced in comparison with that occurring in susceptible plants. Several cell features, including dark blue cytoplasm and altered organelle structure were observed in the central cylinder of *A. stenosperma*, indicating a hypersensitive-like response (HR) of infested host cells. Neither giant cells, nor nematodes developed beyond the second stage, were found on *A. stenosperma*. *Arachis duranensis* showed a delay in the development of nematodes in the roots compared to *A. hypogaea*. The two wild peanut species were chosen to be the contrasting parents of a segregating population for mapping and further investigation of resistance genes.

Keywords: hypersensitive reaction, peanut, resistance, root-knot nematode, susceptibility

Introduction

Root-knot nematodes (*Meloidogyne* spp.) are sedentary endoparasites that have very complex biotrophic relationships with their host plants. Second-stage juveniles (J2s) invade roots in the zone of elongation and then migrate intercellularly and establish a feeding site in the zone of differentiation of the vascular cylinder (Wyss *et al*., 1992; Williamson & Hussey, 1996). *Meloidogyne arenaria* race 1 (Koenning & Barker, 1992; McSorley *et al*., 1992), *M. hapla* (Culbreath *et al*., 1992; Schmitt & Barker, 1998), *M. javanica* (Tomaszewski *et al*., 1994) and *M. haplanaria* (Eisenback *et al*., 2003) are the four described *Meloidogyne* species that parasitize cultivated peanut (*Arachis hypogaea*). The most damaging nematode species for peanut in the USA is *M. arenaria* race 1. Losses caused by this nematode can exceed 50% in severely infested fields (McSorley *et al*., 1992).

Control of root-knot nematodes is difficult, soil nematicides are costly, not always effective and they are detrimental to the environment and human health (Bird & Kaloshian, 2003). Because of this, plant resistance is currently considered the method of choice for controlling root-knot nematodes. Resistance to *M. arenaria* race 1, *M. javanica* and *M. hapla* has been observed in many wild species of peanut (Banks, 1969; Nelson *et al*., 1989; Holbrook & Noe, 1990; Sharma *et al*., 1999). The most damaging of these four species is the peanut root-knot nematode *M. arenaria* race 1. Resistance against this nematode has been characterized for two wild species, *A. cardenasii* and *A. batizocoi*. *Arachis cardenasii* completely inhibited nematode development and resistance was accompanied by a hypersensitive host reaction (Nelson *et al*., 1990). *Arachis batizocoi* caused a reduction in the total number of invading nematodes that reached maturity and produced eggs, and increased the time required for *M. arenaria* to complete its life cycle; no hypersensitive reaction associated with this resistance was observed (Nelson *et al*., 1990). Both species were used to generate resistant *A. hypogaea* cultivars COAN and NemaTAM by a backcross introgression pathway (Simpson, 1991) involving a complex interspecific amphiploid hybrid (Simpson & Starr, 2001; Simpson *et al*., 2003).

In order to identify and characterize new sources of resistance, and to identify contrasting parents that would be suitable for the construction of a population and genetic mapping, an initial screening of a number of wild *Arachis* spp. resistant to *M. arenaria* race 1 was carried out using plants of the *Arachis* Germplasm Bank of EMBRAPA, Brazil. *Arachis stenosperma* accession V10309 and *A. duranensis* accession K7988 were found to be resistant and susceptible parents, respectively. The objectives

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of the present work were: (i) to confirm the resistance or susceptibility of *A. stenosperma* accession V10309 and *A. duranensis* accession K7988, using *A. hypogaea* as a control for susceptibility; (ii) to examine the interaction of *M. arenaria* race 1 with *A. stenosperma, A. duranensis* and *A. hypogaea* cv. IAC-Tatu-ST, to determine when and how resistance is expressed; and (iii) to assess resistance mechanisms to *M. arenaria* race 1 comparing histological responses of the resistant and susceptible *Arachis* spp.

Materials and methods

Nematode inoculum

All experiments were conducted at the EMBRAPA Genetic Resources and Biotechnology quarantine facility with an isolate of *M. arenaria* race 1 from peanut, kindly provided by Dr D. W. Dickson (University of Florida, Gainesville, USA). To confirm the identification, electrophoresis was conducted (Carneiro & Almeida, 2001) and the esterase phenotype A2 was confirmed. Nematodes were multiplied first on peanut (*A. hypogaea* cv. IAC-Tatu-ST) to select a virulent population, and then multiplied afterwards on tomato (*Solanum esculentum* cv. Santa Cruz) for 3 months. Eggs were extracted from infected roots using 0·5% NaOCl (Hussey & Barker, 1973). Second-stage juveniles (J2s) were collected from the root and egg suspension, using modified Baermann funnels. Eggs or freshly hatched J2s were collected by centrifugation and counted using Peters slides under a light microscope (Vrain, 1977).

Nematode reproductive behaviour

Two wild species of *Arachis* (*A. stenosperma* accession V10309 and *A. duranensis* accession K7988) were evaluated under greenhouse conditions (25–30°C) for resistance to *Meloidogyne arenaria* race 1. The susceptible commercial *A. hypogaea* cv. IAC-Tatu-ST was used as a control. Plants were grown from seed in plastic pots (3000 cm^3 volume) containing a moist, steam-sterilized soil (85% sand, 10% silt and 5% clay). When plants were 4 weeks old, they were inoculated with 10 000 eggs per plant (Pi = initial inoculum level), extracted from infected tomatoes using 0·5% NaOCl solution following Hussey & Barker's method (1973), but using a blender instead of manual agitation. Nematode eggs and J2s were introduced into 3-cm-deep holes around the collar region of the plant. The pots were arranged in a complete randomized block design with eight replications. Seventy-five days after plant inoculation (DAI), the different treatments were evaluated by extracting eggs and J2 from the entire root system, using 1% NaOCl with a blender as previously indicated. The final population density (Pf) was quantified using a Peters slide under the microscope and the nematode reproductive factor (RF = Pf/Pi) was calculated (Oostenbrink, 1966). Average reproduction factors, using the transformation by log $(x + 1)$, were compared by the Tukey test with significant difference at the 5% probability level. Treatments with RF < 1·00 were considered as resistant to *M. arenaria* and those with RF > 1·0 as susceptible (Oostenbrink, 1966).

Histopathological studies

Fifteen seedlings with two replicates of each *Arachis* species were transplanted to 200-cm^3 plastic cups containing a sterilized mixture of two parts peat and one part fine sand. Two-week-old plants with six to eight expanded leaves were inoculated with 5000 J2s per plant. Distilled water without nematodes was added to control plants. Roots were removed from the cups and carefully washed at the following sampling times: 3, 4, 6, 8, 9, 11, 13, 15, 16, 17, 19, 32, 48 and 63 days after inoculation (DAI). Several root tips (1- to 3-cm segments) were analysed using stereomicroscopy and light microscopy (Axiophoto, Zeiss) using the technique for clearing (NaOCl) and staining (acid fuchsin) plant tissues described by Byrd *et al.* (1983). This technique was used to facilitate localization of the infection sites. For fine cuttings, root-tips with swellings were excised and fixed in 2% glutaraldehyde in 0·1 M sodium cacodylate buffer pH 7·0 at 4°C for 48 h. They were rinsed three times for 10 min in 0·1 M sodium cacodylate buffer pH 7·0, post-fixed in 2% osmium tetroxide in 0·1 M sodium cacodylate buffer pH 7·0 for 2 h, and rinsed again in the same buffer three times for 5 min each. After dehydration in a graded series of ethanol (20–100%) followed by two additional steps in absolute ethanol, samples were infiltrated with increasing volumes of Spurr's resin (1v:3v; 2v:3v; 3v:3v; 4v:3v) in a constant volume of 100% ethanol for 4–6 h and polymerized at 70°C for 24 h. Embedded tissues were cut into 0·5- to 1·4-μm-thick sections with an ultramicrotome and the sections were placed on glass slides, stained with a drop of toluidine blue (1% w/v in borax, pH 8·9) and dried with a flame. The sections were examined with the light microscope (Axiophoto, Zeiss).

Results

Reproduction of *Meloidogyne arenaria* race 1 in *Arachis* spp.

The reproductive behaviour (RF) of *M. arenaria* race 1 at 120 DAI (Table 1) in three species of *Arachis* showed that the control *A. hypogaea* cv. IAC-Tatu-ST was considered susceptible (RF = 16·4), *A. duranensis,* (K7988) moderately susceptible (RF = 3·5) and *A. stenosperma* (V10309) highly or completely resistant (RF = 0). These results were in accordance with the objectives of characterizing the contrasted response in wild species challenged with nematodes (Table 1).

Histological observations of infected susceptible *A. hypogaea* cv. IAC-Tatu-ST and *A. duranensis* K7988

At 3–8 DAI, macroscopic observation of cleared root tissue stained with acid fuchsin revealed many J2s located

Table 1 Reproduction of *Meloidogyne arenaria* race 1 on *Arachis* spp. accessions in a greenhouse test

Arachis spp.	Fresh root weight (g)	Nematodes g^{-1} fresh root weight	Reproduction factor[a]
A. stenosperma (V10309)	8·7	0	0 c (R)
A. duranensis (K7988)	17·0	1781	3·5 b (MS)
A. hypogaea cv. IAC-Tatu-ST	11·7	13811	16·4 a (S)

[a]Numbers with different lowercase letters differ from one another by the Tukey test at the 5% level. S = susceptible, MS = moderately susceptible, R = resistant.

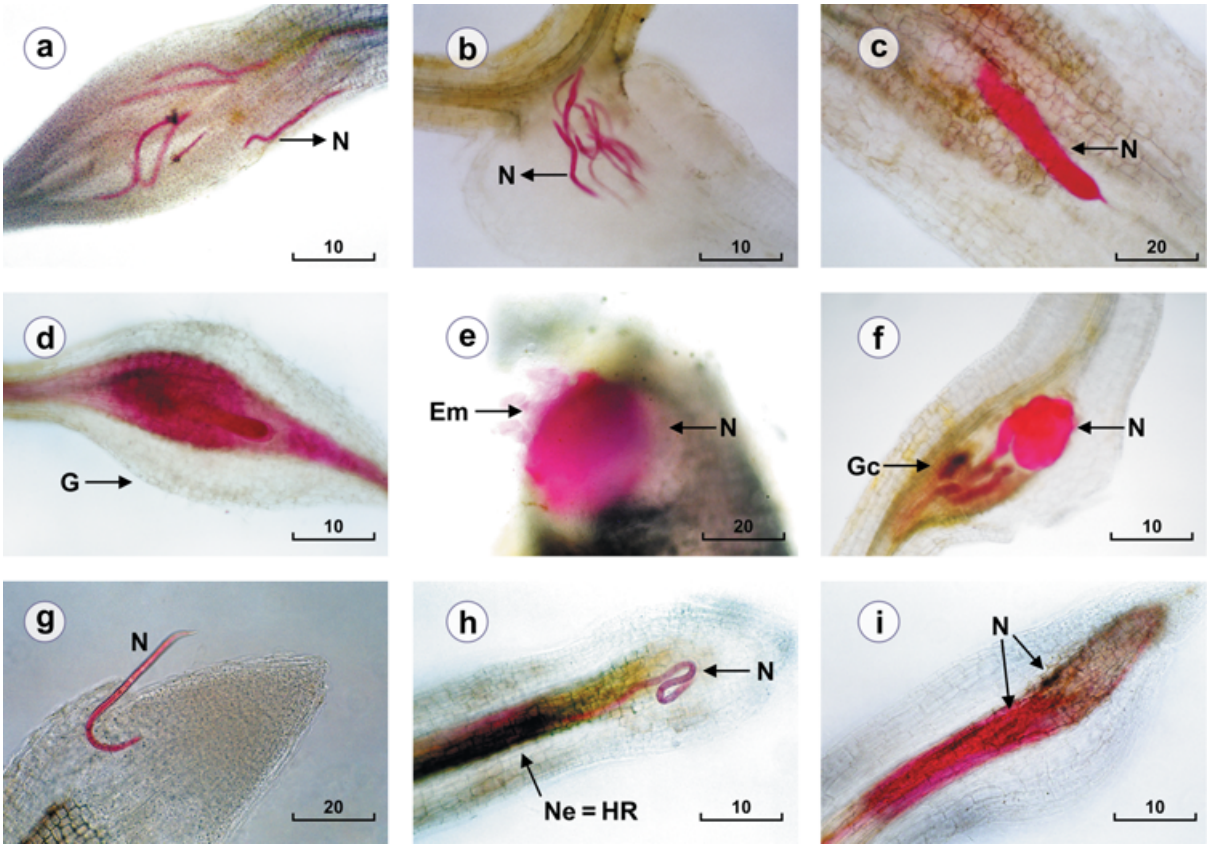

Figure 1 Acid-fuchsin preparations of the development of *Meloidogyne arenaria* on infected roots of cultivated peanut *Arachis hypogaea* cv. IAC-Tatu-ST (b, 6 DAI; d, 11 DAI; e, 32 DAI), wild peanut *A. duranensis* accession K7988 (a, 8 DAI; c, 11 DAI; f, 63 DAI) and wild peanut *A. stenosperma* accession V10309 (g, 8 DAI; h, 17 DAI; I, 19 DAI). N = nematode, Ne = necrotic lesion, HR = hypersensitive reaction, EM = egg masses, Gc = giant cells. Scale bars in μm.

in roots, suggesting successful penetration of roots through the apical region in both species, and migration in groups through the cortex (Fig. 1a and b). Similar features were still observed until 11 DAI. At 9 DAI, swollen J2/J3s were seen in the vascular cylinder where they are thought to initiate the establishment of the feeding site. At 11–13 DAI, infected roots began to enlarge in both peanut species; J3s were observed in *A. duranensis* with slightly swollen roots (Fig. 1c) and J3/J4s in *A. hypogaea* roots forming large galls at the nematode infection sites (Fig. 1d). At 15–19 DAI, macroscopic observation showed that in both species feeding sites had already been established. At 32 and 48 DAI females with egg masses occurred in *A. hypogaea*, while at 63 DAI, in *A. duranensis* roots globose females were still seen (Fig. 1e and f). The development of *M. arenaria* race 1 in *A. hypogaea* roots was faster than in *A duranensis*.

Microscopic analysis clearly showed the delay in nematode development in the moderately susceptible wild species, *A. duranensis*, as compared to that in the susceptible cultivated peanut. In both species, nematodes were localized close to vessels, indicating that the formation of galls was initiated. *Arachis hypogaea* host cells in front of the nematode's anterior region showed increased size at 8 DAI (Fig. 2a), predicting their final step of differentiation to giant cells. The giant cells formed feeding sites with a

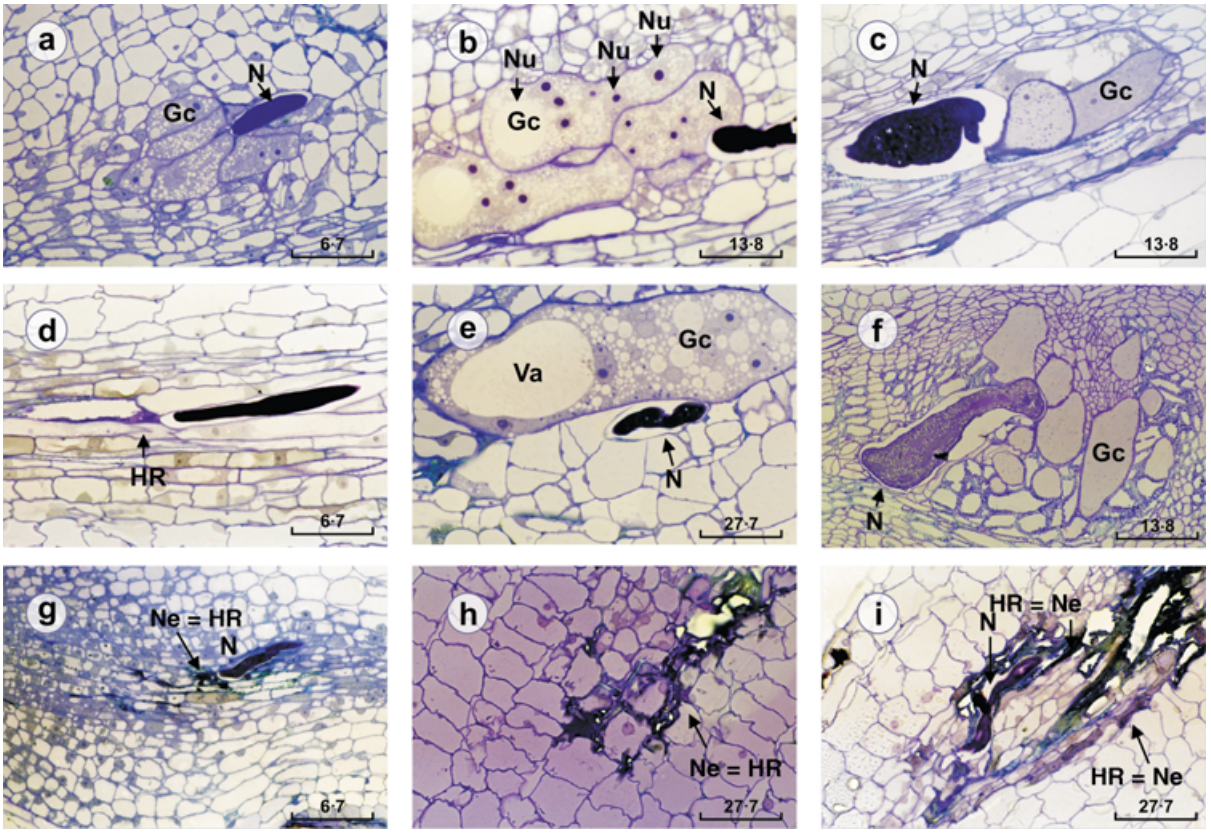

Figure 2 Longitudinal sections of roots of *Arachis hypogaea*, *A. duranensis* and *A. stenosperma* infected with *Meloidogyne arenaria* race 1. Sections were stained with toluidine blue and show the timing of the nematode infection process in the susceptible species *A. hypogaea* (a) 8 DAI, (b) 16 DAI (arrows show vacuoles and nucleoles) and (c) 19 DAI; *A. duranensis* (d) 16 DAI (arrow shows thickening of the cell wall), (e) 19 DAI and (f) 32 DAI; *A. stenosperma* (g) 8 DAI, (h) 16 DAI and (i) 19 DAI. N = nematode, Ne = necrotic lesion, HR = hypersensitive reaction, NU = nucleous, Va = vacuoles, Gc = giant cells. Scale bars in μm.

regular oval shape, each one containing one large vacuole, several small vacuoles and some small nuclei (three to nine per giant cell) at 16 DAI (Fig. 2b) and at 19 DAI (Fig. 2c). In *A. duranensis*, however, it was only at 16 DAI that nematodes were seen associated with the presence of giant cells. At 16 DAI, cell walls showed increase in thickness to form ingrowths (Fig. 2d). From 19 DAI, giant cells contained many vacuoles (Fig. 2e), reaching their final stage of development at 32 DAI. In *A. duranensis*, giant cells also presented an oval shape and multiple nuclei (Fig. 2f), but it appeared that more giant cells were induced (eight to ten), compared with four to six in *A. hypogaea*.

Histopathological observations of infected resistant wild peanut, *A. stenosperma* V10309

At 3–9 DAI, macroscopic observation revealed a reduced number of J2 in the sub-apical region (Fig. 1g) compared with the distribution of nematodes in infected susceptible roots. Nematodes were observed at the root surface with their anterior regions located in the first cell layers, indicating that penetration was in progress until 17 and 19 DAI (Fig. 1h and i, respectively). Close to the anterior region of nematodes localized inside the vascular cylinder of the roots, host cells stained with acid fuchsin showed a dark yellow-brown colour, indicating that induction of feeding sites by the nematode was associated a necrotic-like response (Fig. 1h). The J2s at these sites of necrosis were brown, indicating that they may have died, it is possible that the whole region around the nematode was oxidizing (Fig. 1i). Macroscopic examination at 8–19 DAI demonstrated that J2s took about this time to penetrate and migrate from the root tip through the cortex into the developing vascular cylinder. The HR apparently never occurred in the cortex region during the early stages of invasion. Every J2 found in the vascular cylinder was associated with a hypersensitive reaction.

Microscopic analysis at 8 DAI showed J2s located in the vascular cylinder. Close to the anterior portion of the nematode, host cells showed a dark-blue-stained and collapsed cytoplasm in which nuclei were not visible (Fig. 2g). Most of the nematodes observed also displayed a disorganized cellular content showing accumulation of dark-blue-stained material (data not shown). Observations of thin sections showed J2s in the epidermis, cortex and vascular region and embedded tissues stained with toluidine blue displayed dark blue staining at the necrosis-like sites (Fig. 2h). No swollen nematodes or feeding sites were

established in infected roots and consequently no giant cell was induced in any time sample analysed. Hypertrophy of the tissue or hyperplasia of the cells were never observed. The hypersensitive reaction occurred around cells of the nematode's anterior region in the vascular cylinder and never in cortex or epidermis cells (Fig. 2i). No nematodes were found beyond 19 DAI, although some degraded and necrotic roots were found 32–63 DAI.

Discussion

This study presents a histological time course study of the development of the infective cycle of *M. arenaria* race 1 in roots of two wild species of peanut, *A. stenosperma* and *A. duranensis*, and the cultivated peanut, *A. hypogaea* cv. IAC-Tatu-ST. First, this work aimed to confirm the contrasting behaviour of two wild *Arachis* species with regard to *M. arenaria* race 1 resistance and to compare them with the commercial *A. hypogaea* cv. IAC-Tatu-ST. Secondly, this study analysed the penetration and subsequent development of the root-knot nematode in these peanut species.

Arachis hypogaea cv. IAC-Tatu-ST is one of the most traditionally cultivated peanut varieties in Brazil and it was chosen as a susceptible control for *M. arenaria* race 1. In terms of the reproduction factor (RF), the Brazilian commercial peanut (RF = 16·4) was similar to the American commercial cv. Florunner (RF = 15·496), one of most cultivated peanuts in the USA (Holbrook *et al*., 2000).

Although *A. duranensis* K7988 had a lower reproduction factor (RF = 3·5) than that evaluated in *A. hypogaea* cv. IAC-Tatu-ST, it was considered susceptible according to the Oostenbrink (1966) or Hussey & Janssen (2002) concepts (only plants with RF < 1 or < 10% of the replication in susceptible genotypes, respectively, are considered resistant). It was demonstrated that *A. stenosperma* V10309 had a high level of resistance, showing no nematode reproduction (RF = 0) (Table 1).

Second-stage juveniles of *M. arenaria* race 1 penetrated roots of the susceptible wild species *A. duranensis*, established feeding sites and induced giant cells in the same way as in the cultivated species. Nevertheless, there were some differences: (i) only 32 days were required for a complete life cycle in *A. hypogaea*, compared with over 63 days in *A. duranensis*; (ii) galls were small in *A. duranensis* and large in *A. hypogaea*; and (iii) giant cells were larger and higher in number in *A. duranensis* (8–10) than in *A. hypogaea* (4–6). Thus, it seems that host plant species affects these features. Vovlas *et al*. (2005) studied the infection of chickpea by different *Meloidogyne* spp. and suggested that the size of galls and size and number of giant cells were influenced by host plant species rather than *Meloidogyne* species.

The data found suggest that *A. stenosperma* displays at least two kinds of resistance to *M. arenaria* race 1. One kind of resistance suppressed root penetration by J2s and the other kind blocked nematode development after penetration. Failure of J2s to penetrate the resistant *A. stenosperma* may indicate physical or chemical root barriers. Such barriers were suggested previously for resistant grape rootstock (Anwar & McKenry, 2002), cotton (Anwar *et al*., 1994), soyabean (Dropkin & Nelson, 1960), pepper (Pegard *et al*., 2005) and coffee cultivars (Anthony *et al*., 2005). It may also indicate that the root exudates did not attract or perhaps even repelled the J2s, or that the J2s penetrated and then left the roots. Such protection was shown for *Cucumis sativus*, where a triterpene, cucurbitacin, which repelled *Meloidogyne* J2s, was isolated from root exudates (Hayene & Jones, 1976). Similarly, amino acids exuded from *Sesamum indicum* roots had a nematostatic effect on *Meloidogyne* J2s (Tanda *et al*., 1989).

No differences in root penetration by the nematode, between susceptible and resistant roots were reported for two wild *Arachis* species, *A. cardenasii* and *A. batizocoi*, both resistant to *M. arenaria* race 1 (Nelson *et al*., 1990). This was also observed in soyabean (*Glycine max*; Herman *et al*., 1991) and common bean, (*Phaseolus vulgaris*; Sydenham *et al*., 1996).

Moreover, in the resistant species *A. stenosperma*, the J2s that did penetrate remained vermiform and clustered in vascular cylinder cells; no development, no galling and no egg masses were observed. This suggested that this wild peanut also has a very active post-penetration biochemical defence mechanism which blocked nematode development and reproduction. The post-penetration response of the incompatible *A. stenosperma* roots was considered a classical HR, which occurred after 8–19 DAI. This is in line with several reports on other plants in which HR was correlated with resistance to nematodes (Dropkin, 1969; Bleve-Zacheo *et al*., 1998; Rodrigues *et al*., 2000; Anthony *et al*., 2005; Pegard *et al*., 2005). However, the differentiation time of HR differs between plants, occurring hours after penetration for some, and only after days for others. It has been proposed that early HR occurring in the outer tissues of the root (i.e. the epidermis and root cortex) and immediately after penetration (12–48 h) would be relatively easily overcome by newly virulent nematodes (Bleve-Zacheo *et al*., 1998; Castagnone-Sereno *et al*., 2001). Instead, if the HR occurs later and deeper in the root (i.e. close to or within the vascular cylinder) this would irreversibly block any further nematode development, making the selection of new virulent genotypes less likely. The HR was also the mechanism of nematode resistance for the wild *A. cardenasii* (Nelson *et al*., 1990) and, similarly to *A. stenosperma*, it was a late HR, occurring 7 days post-inoculation.

In order to enable the mapping of genes responsible for nematode resistance, *A. stenosperma* and *A. duranensis* were used as resistant and susceptible parentals to produce a segregating F_2/F_3 population, and this population was used to make the first microsatellite-based genetic map in *Arachis* (Moretzsohn *et al*., 2005). Bioassays in the F_3 generation will allow the mapping of the R-genes, although these bioassays have not yet been carried out because of the difficulties of performing a large bioassay under quarantine conditions. In a parallel approach to identify genes responsive to nematode challenge, ESTs were generated from inoculated and control roots of

A. stenosperma (Proite *et al*., 2007). Analysis of the genes expressed in control and inoculated roots will generate useful information to target genes closely associated with the resistance of peanut to nematode.

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