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New sources of resistance to *Meloidogyne incognita* race 3 in wild cotton accessions and histological characterization of the defence mechanisms

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Accessions of *Gossypium* spp., some of them never previously tested, were evaluated for resistance to a local isolate of *Meloidogyne incognita* race 3 in greenhouse experiments. Nematode infection was characterized based on the galling and egg mass indexes and the reproduction factors (RF). Root-knot nematode reproduction among the newly tested accessions varied from highly susceptible (AS0188, AS0189) to moderately resistant (MT123 no. 3), and some accessions showed highly reduced nematode reproduction (CIR1343, CIR1348, Fai Mui). Histological observations of two resistant accessions (*G. barbadense* CIR1348 and *G. hirsutum* TX-25, respectively) showed that resistance occurs through a two-stage mechanism in the first accession and through a single-stage mechanism in the second. Parasitism is blocked early after second-stage juvenile (J2) penetration or during its initial tissue migration (CIR1348) and the development of later-stage juveniles into female adults is suppressed at a later stage (TX-25 and CIR1348). Fluorescence and bright light microscopy showed that root cells surrounding nematodes exhibit a hypersensitivity-like reaction, with the accumulation of presumably phenolic compounds and the presence of necrotic cells that limit the development of nematodes and the formation of giant cells. Underdeveloped giant cells with degenerated cytoplasmic content were found in small numbers in CIR1348 and in large numbers in TX-25, along with deformed nematodes. The full characterization of the defence mechanisms of novel sources of resistance to the root-knot nematode in cotton constitutes a first step towards their use in crop improvement.

Keywords: *Gossypium* spp., host plant resistance, hypersensitive response, *Meloidogyne incognita*

Introduction

The root-knot nematode (RKN) *Meloidogyne incognita* is a severe pathogen of cotton, causing direct damage and increasing the severity of other root diseases, in particular the fusarium wilt disease caused by *Fusarium oxysporum* f. sp. *vasinfectum*. Plant resistance and the development of resistant varieties have the potential to contribute to efficient RKN management in cotton. Resistant varieties help to control the disease and maintain crop yield, with a significant long-term impact by decreasing nematode populations in the soil and protecting following crop rotations. In this respect, the identification and full characterization of effective sources of resistance is of great interest.

A search for high levels of RKN resistance in cotton germplasm has been undertaken over the years, in cultivated species as well as in wild relatives (Robinson *et al.*, 2004). Despite these efforts, few accessions with a high level of resistance have been identified. In the earlier studies, none of the accessions reached the level of resistance of Auburn 623 RNR, or its equally resistant derivative Auburn 634 RNR. In a more recent study, Robinson *et al.* (2004) identified three accessions of *Gossypium hirsutum* (TX-25, TX-1828 and TX-1860) that showed resistance levels equivalent to that of Auburn 623 RNR. This elite breeding line (Shepherd, 1974a), which was selected from crossing between two moderately resistant accessions, Clevewilt-6 and Wild Mexican Jack Jones (Shepherd, 1974b), exhibits the highest level of resistance to RKN known to date in cotton, and has been used to

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derive a number of breeding lines (Shepherd *et al*., 1996). Nevertheless, the high level of resistance of Auburn 623 RNR and of its derivatives ('M-series') has never been transferred to superior cultivars. The cultivar Clevewilt-6 is also at the origin of the now obsolete varieties Stoneville LA 887 (Jones *et al*., 1991) and Paymaster (Hartz) 1560, which were widely cultivated for their moderate levels of resistance to RKN, and of their sibling lines (La. RN 4-4, La. RN 909, La. RN 910, La. RN 1032; Jones *et al*., 1988). To date, the only available moderately RKN-resistant varieties with desirable agronomic and quality standards have been Acala Nem X (Oakley, 1995) and Acala Nem X H Y (Anonymous, 2005), which have a restricted area of adaptation due to their particular characteristics ('Acala-type cotton').

Variability in the virulence of RKN isolates on resistant cotton genotypes has been demonstrated (Zhou *et al*., 2000). Furthermore, selection of isolates with increased reproduction on resistant varieties after repeated exposures to resistant cotton was also reported (Ogallo *et al*., 1997), indicating the continuing need to increase the number of sources with effective durable resistance. Indeed, cotton breeding for RKN resistance presently relies on a few sources of resistance, making such genotypes vulnerable to resistance breakdown.

This work presents results of the screening of cotton genetic resources to identify novel sources of resistance to the root-knot nematode. This screening allowed the identification of cotton accessions with high levels of resistance, equalling that of the most resistant lines (M-315 RNR, a derivative of Auburn 623 RNR) known to date. Histological characterization of the resistance mechanism in the *Gossypium barbadense* new accession CIR1348 and *G. hirsutum* accession TX-25 showed that resistance may result from two distinct responses that act in isolation or jointly to suppress gall formation and nematode reproduction.

Material and methods

Gossypium genotypes

Gossypium hirsutum, G. barbadense and *Gossypium arboreum* accessions used in this study came from either the Cirad or Embrapa germplasm collections (Table 1). Some of these genotypes have been previously tested, and include modern or obsolete cultivars, breeding lines and wild accessions. *Gossypium hirsutum* cv. FiberMax966 (FM966) was used as a susceptible check, while *G. hirsutum* breeding line M-315 RNR was used as a resistant control.

Nematode inoculation

A *M. incognita* race 3 population collected in Londrina (State of Paraná, Brazil) was used in this study due to its pathogenicity on cotton. Identification of the species was done using the Esterase (Est) phenotype (Carneiro & Almeida, 2001) and SCAR markers (Randig *et al*., 2002), and the race was determined according to Hartman & Sasser (1985).

Prior to inoculation, the population was multiplied on tomato (*Solanum lycopersicum* cv. Santa Clara) for 3 months under

Table 1 Description of the *Gossypium* spp. accessions used in the study

Accession name	Species	Origin – accession number
AS0110	*G. hirsutum* race marie-galante	Haiti – wild accession; Cirad accession no. CIR6098; USDA accession TX-1828, NPGS PI 530459
AS0188*	*G. hirsutum* race marie-galante	Guadeloupe – wild accessions; Cirad accession no. CIR6172
AS0189*	*G. hirsutum* race marie-galante	Guadeloupe – wild accession; Cirad accession no. CIR6173
AS0190	*G. hirsutum* race marie-galante	Guadeloupe – wild accession; Cirad accession no. CIR6174; USDA accession TX-1860, NPGS PI 530491
AS0191*	*G. hirsutum* race marie-galante	Guadeloupe – wild accession; Cirad accession no. CIR6175
Auburn 56	*G. hirsutum*	USA – obsolete cultivar with resistance to fusarium wilt disease
CIR1343*	*G. barbadense* race barbadense	Peru – wild accession; Cirad accession no. CIR1343
CIR1348*	*G. barbadense* race barbadense	Peru – wild accession; Cirad accessions no. CIR1348
China 13–9*	*G. arboreum*	China – obsolete cultivar; Cirad accession no. CIR1550
Clevewilt-6	*G. hirsutum*	USA – obsolete cultivar with moderate resistance to RKN
DeltaOpal	*G. hirsutum*	Australia – commercial cultivar
Deltapine 61	*G. hirsutum*	USA – obsolete cultivar
Fai Mui*	*G. arboreum*	Laos – obsolete cultivar; Cirad accession no. CIR1549
Fibermax966 (FM966)	*G. hirsutum*	Australia – commercial cultivar
La. RN-1032	*G. hirsutum*	USA – breeding line with resistance to RKN
LA-887	*G. hirsutum*	USA – obsolete cultivar with resistance to RKN
M-315 RNR	*G. hirsutum*	USA – breeding line highly resistant to RKN
MT121 Bulk no. 6*	*G. barbadense* race brasiliensis	Brazil – wild accession
MT123 no. 3*	*G. barbadense* race brasiliensis	Brazil – wild accession
TX-25	*G. hirsutum* race punctatum	Mexico – wild accession; NPGS PI no. 154035
VH8-4602*	*G. barbadense* race barbadense	Antigua – obsolete cultivar; Cirad accession no. CIR1286
Wild Mexican Jack Jones (WMJJ)	*G. hirsutum*	Mexico – wild accession; USDA accession TX-2516, NPGS PI no. 593649

*Previously untested accessions.

greenhouse conditions. Eggs were extracted from infected roots using 0·5% NaOCl according to Boneti & Ferraz (1981). For histopathological studies, freshly hatched second-stage juveniles (J2) were collected using modified Baermann funnels. In both cases, counting was done using a light microscope and Peters slides.

Evaluation of nematode resistance

Eight plants of each genotype were grown in pots 20 cm high and 15 cm diameter filled with a mixture (1:1) of autoclaved soil and Plantimax® compost, in a greenhouse maintained at 22–28°C. Thirty days after seed emergence, pots were inoculated with 5000 (first assay) and 10 000 (second assay) *M. incognita* race 3 eggs by pipetting the nematode suspension around the stem base. In the second assay, heavier inoculums were used to confirm the results of the previous test using more severe conditions. Plants, arranged in a randomized block design with eight replications, were maintained in the greenhouse at a temperature ranging between 25 and 30°C, and were watered and fertilized as necessary. One hundred and twenty days after inoculation (dai), plants were uprooted, the root system rinsed under tap water, and the roots weighed. Roots were stained with phloxin B and evaluated for gall and egg mass numbers (galling index, GI; egg mass index, EMI), using a scale where 0 = no galls or egg masses; 1 = 1–2 galls or egg masses; 2 = 3–10 galls or egg masses; 3 = 11–30 galls or egg masses; 4 = 31–100 galls or egg masses; and 5 = >100 galls or egg masses per root system (Hartman & Sasser, 1985).

Eggs were extracted using Boneti & Ferraz's (1981) modified extraction method using 1% NaOCl as described above, and the total egg number per plant was calculated. The reproduction factor (RF) was calculated as RF = FP/IP, where FP = final nematode population and IP = initial nematode population (I = 5000 or 10 000). The average RF was transformed to $\log_{10}(x + 1)$ and, after analysis of variance, the averages were compared using Scott–Knot's test at the 5% probability level. Accessions were classified as highly susceptible (HS), susceptible (S), moderately resistant (MR), resistant (R) or highly resistant (HR) according to the statistical analysis and Roberts' (2002) concepts.

Histopathological observations

Plantlets of accession CIR1348, TX-25 and the susceptible check FiberMax966 were grown in plastic cups containing washed and sterilized sand, and fertilized weekly. Plantlets were inoculated with 10 000 J2s of *M. incognita* race 3 per plant. Plantlets, three resistant and three susceptible per time point, were carefully removed from the cups at 2, 4, 7, 9, 11, 16, 18, 21, 23, 28, 34 and 45 dai and their roots rinsed with water.

Some of the roots from susceptible and resistant plants were stained with acid fuchsin as described by Byrd *et al.* (1983) 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).

Other parts of the roots were embedded in resin to produce thin sections. Root fragments showing galls or swelling, 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.

Results

Resistance of cotton accessions to *M. incognita* race 3

Resistance was evaluated based on three criteria: galling index, egg mass index and reproduction factor (RF). Results are shown in Tables 2 and 3. No difference was observed between the two levels of inoculum used (5000 or 10 000 eggs per plant) with respect to phenotype. As expected, nematodes failed to reproduce on the resistant control M-315 RNR. Furthermore, gall and egg mass formation were also suppressed. In contrast, the susceptible check FM966 exhibited high gall

Table 2 Mean galling index (GI), egg mass index (EMI), total number of eggs per gram fresh roots and reproduction factor (RF) presented by the different *Gossypium* spp. 120 days after inoculation with 5000 *Meloidogyne incognita* eggs per plant. Eight plants per accession were scored 120 days after inoculation. The phenotype is also indicated

Accession	GI[a]	EMI[a]	Eggs g^{-1} fresh roots	RF[b]	Phenotype[c]
AS0110	4·8	4·6	1734	21·44 a	HS
AS0188*	3·1	4·9	1359	23·97 a	HS
AS0189*	4·0	5·0	2127	21·81 a	HS
AS0190	5·0	5·0	7602	60·71 a	HS
AS0191*	3·3	3·8	695	12·18 b	S
Auburn 56	1·9	1·1	45	0·20 d	R
CIR1343*	1·5	1·0	22	0·19 d	R
CIR1348*	0	0	1	0·01 d	HR
China 13–9*	3·6	3·3	990	8·28 b	S
Clevewilt-6	3·3	2·6	420	2·58 c	MR
Delta Opal	3·4	2·5	321	3·19 c	MR
Deltapine 61	4·0	3·6	566	7·54 b	S
Fai Mui*	1·4	1·7	106	0·48 d	R
FM966	5·0	5·0	1994	14·05 b	S
La. RN-1032	4·1	2·1	246	1·11 c	MR
LA-887	4·2	2·0	81	1·10 c	MR
M-315 RNR	0·8	0·0	4	0·03 d	HR
MT121 Bulk no. 6*	4·9	3·3	607	11·12 b	S
MT123 no. 3*	3·1	2·1	176	2·87 c	MR
VH8-4602*	4·1	4·1	636	7·66 b	S
TX-25	3·0	0·4	4	0·03 d	HR
Wild Mexican Jack Jones	0·5	0·3	3	0·03 d	HR

*Previously untested accessions.

[a]Mean value of GI or EMI. 0 = no galls, 1 = 1–2 galls, 2 = 3–10 galls, 3 = 11–30 galls, 4 = 31–100 galls, and 5 = >100 galls per root system (Hartman & Sasser, 1985).

[b]Mean values are transformed in log ($x + 1$). Means followed by different letters are significantly different ($P < 0·05$) according to Scott–Knot's test.

[c]HS = highly susceptible, S = susceptible, MR = moderately resistant, R = resistant, HR = highly resistant.

Table 3 Mean galling index (GI), egg mass index (EMI), total number of eggs per gram fresh roots and reproduction factor (RF) presented by the different *Gossypium* spp. 120 days after inoculation with 10 000 *Meloidogyne incognita* eggs per plant. Eight plants per accession were scored 120 days after inoculation. The phenotype is also indicated

Accession	GI[a]	EMI[a]	Eggs g^{-1} fresh roots	RF[b]	Phenotype[c]
AS0110	5·0	4·8	4163	28·52 a	HS
AS0188*	5·0	4·8	3065	29·37 a	HS
AS0189*	5·0	5·0	2127	27·18 a	HS
AS0190	5·0	5·0	7602	32·14 a	HS
AS0191*	4·0	4·2	1674	14·28 b	S
Auburn 56	2·0	1·4	201	0·59 d	R
CIR1343*	0·8	1·2	47	0·22 d	R
CIR1348*	0·0	0·0	9	0·05 d	HR
China 13–9*	3·9	3·5	1375	6·53 b	S
Clevewilt-6	3·3	2·6	434	1·68 c	MR
Delta Opal	3·8	2·7	513	2·89 c	MR
Deltapine 61	4·0	4·2	1224	6·89 b	S
Fai Mui*	1·2	1·5	137	0·39 d	R
FM966	5·0	5·0	5552	24·15 b	S
La. RN-1032	2·8	2·1	253	1·32 c	MR
LA-887	2·2	2·0	139	1·05 c	MR
M-315 RNR	0·0	0·0	2	0·01 d	HR
MT121 Bulk no. 6*	5·0	3·8	1638	15·14 b	S
MT123 no. 3*	2·9	2·0	346	2·51 c	MR
VH8-4602*	4·5	4·3	1461	8·52 b	S
TX-25	3·5	0·5	5	0·04 d	HR
Wild Mexican Jack Jones	0·4	0·2	2	0·01 d	HR

*Previously untested accessions.

[a]Mean value of GI or EMI. 0 = no galls, 1 = 1–2 galls, 2 = 3–10 galls, 3 = 11–30 galls, 4 = 31–100 galls, and 5 = >100 galls per root system (Hartman & Sasser, 1985).

[b]Mean values are transformed in log ($x + 1$). Means followed by different letters are significantly different ($P < 0·05$) according to Scott–Knot's test.

[c]HS = highly susceptible, S = susceptible, MR = moderately resistant, R = resistant, HR = highly resistant.

and egg mass numbers and a high level of nematode reproduction.

Accessions that had previously been tested exhibited resistance reactions consistent with previous findings, ranging from resistant (Auburn 56, TX-25, Wild Mexican Jack Jones) to moderately resistant (LA 887, La. RN-1032, Delta Opal and Clevewilt-6). As expected, Deltapine 61 was susceptible.

All *G. hirsutum* race marie-galante accessions supported high levels of nematode reproduction, and were classified as highly susceptible (AS0110, AS0188, AS 0189 and AS0190) or susceptible (AS0191). One of the two diploid cotton varieties tested (China 13–9) was susceptible, as was the *G. barbadense* variety VH8-4602 and the *G. barbadense* wild accession MT121 Bulk no. 6. The second *G. barbadense* wild entry (MT123 no. 3) was classified as moderately resistant. Resistant accessions (RF < 1) included the two Peruvian wild *G. barbadense* entries (accessions CIR1348 and CIR1343) as well as the obsolete Lao diploid cultivar Fai Mui (Tables 2 & 3). The resistant accessions, in addition to showing reduced nematode reproduction, exhibited low GIs and EMI (0–2). An exception to this rule was accession TX-25, which had a GI of 3 and EMI = 0.

Histological observations of cotton–*Meloidogyne incognita* race 3 interactions

The susceptible cultivar FM966 was chosen for the histopathological observations of the compatible reaction. Examination of acid fuchsin-stained roots and toluidine blue-stained sections showed that the J2 were able to penetrate the root tips, migrate along the sieve elements, and develop normally after having initiated the differentiation of feeding sites (Fig. 1a–h). Two days after inoculation, numerous J2s were observed invading the subapical meristem of the roots (Fig. 1a). At 4 dai, J2s present in the root cortex migrated towards the vascular cylinder, thus causing damage to the cell walls (Fig. 1b). At 7 and 9 dai, numerous juveniles were observed within the vascular cylinder, and at 9–16 dai, feeding juveniles were visible inside the vascular cylinder (Fig. 1e). At 11–18 dai, numerous J3/J4 had established feeding sites with 6 to 14 well-formed giant cells containing numerous nuclei, dense cytoplasm, and small vacuoles (Fig. 1f). At that time, enlargement of the vascular cylinder region took place, and small galls were apparent. Giant cells displayed thickened cell walls (Fig. 1g). At 28–34 dai, adult females were observed (Fig. 1h) and their development caused severe injuries to the surrounding cells and disruption of the root cortex. At 45 dai, egg masses were observed at the surface of the infected roots.

The highly resistant *G. barbadense* accession CIR1348 was used to observe the resistance reactions. Microscopical examination of acid fuchsin-stained roots and observation of toluidine blue-stained sections showed that J2s were able to penetrate the epidermis and cortex at 7–9 dai, indicating that penetration was in progress (Fig. 2a,b). Under fluorescence microscopy (UV) and bright field (after toluidine staining) yellow colour and dark-blue regions were revealed, respectively (Fig. 2a,b). At 11–21 dai, J2/J3 were present in the vascular cylinder (Fig. 2c,d). Fluorescence microscopy using UV excitation of root sections harvested at these times showed a strong green or blue autofluorescence around the nematodes in all infection sites examined. The same sections visualized under bright field after toluidine staining revealed dark-blue regions, indicating major alteration of cells (cell death) in contact with the nematodes (Fig. 2c–f). Lesions typical of an hypersensitive response (HR) were found around all nematodes when they penetrated the epidermis and migrated through the cortex or when they reached the vascular cylinder (Fig. 2a–f). In an HR, programmed cell death occurs around the area of infection and the development of the pathogen is arrested. Longitudinal sections also showed almost entire bodies of nematodes completely sur-

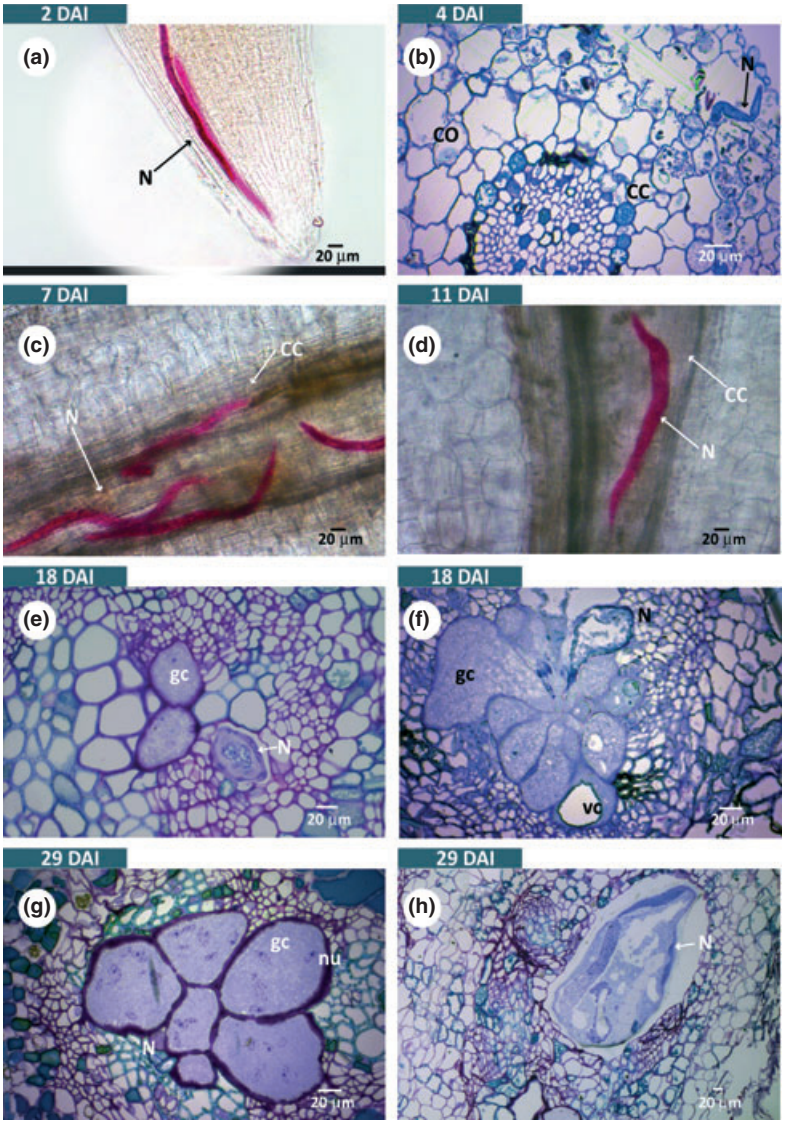

Figure 1 Susceptible interaction. Roots of *Gossypium hirsutum* accession FM966 (susceptible control) infested with *Meloidogyne incognita* race 3. Bright field microscopical observations of root fragments stained with acid fuchsin (a,c,d) and sections stained with toluidine blue (b,e,f,g,h). (a) Second stage juveniles (J2) inside the root tip; (b) intercellular localization of a J2 in the root cortex; (c) several J2 individuals within the central cylinder; (d) J2 after feeding inside the central cylinder; (e), gall with a nematode surrounded by giant cells showing dense cytoplasm and nucleus; (f) a feeding site with J3/J4 nematodes inside and vacuoles; (g) giant cells exhibit dense cytoplasm and thickened cell walls which are stained dark-blue; (h) female showing developed ovary. N = nematode, gc = giant cells, CC = central cylinder, CO = cortex, vc = vacuole, nu = nucleus.

rounded by autofluorescence or toluidine dark-stained components (Fig. 2a–f).

At 21–29 dai, few giant cells were observed, some of them showing multiple nuclei, and reduced thickening of walls (Fig. 2g). At 21 dai, markedly deformed J3/J4 were detected in the vicinity of the altered giant cells (Fig. 2g). At 29–34 dai, most giant cells had degenerated, and had retracted cytoplasm containing numerous vacuoles (Fig. 2h). No adult females with eggs were seen in any of the 34–45 dai sections analysed.

External observations of the roots at 30–45 dai revealed large well-developed galls in susceptible roots, whereas the resistant roots supported only some small residual swelling around the feeding sites. Acid fuchsin staining at 29–45 dai revealed that, in susceptible FM966 roots, the females had reached reproductive maturity and started to lay eggs, whereas in resistant roots, the J3/J4 or under developed female nematodes showed no sign of egg production. This confirmed the observations of screening accessions (Tables 2 & 3).

The resistant *G. hirsutum* accession TX-25 did not show an HR response to nematode penetration at early infection (Fig. 3a). However, when roots were examined at 14 dai, visible evidence of an HR response was

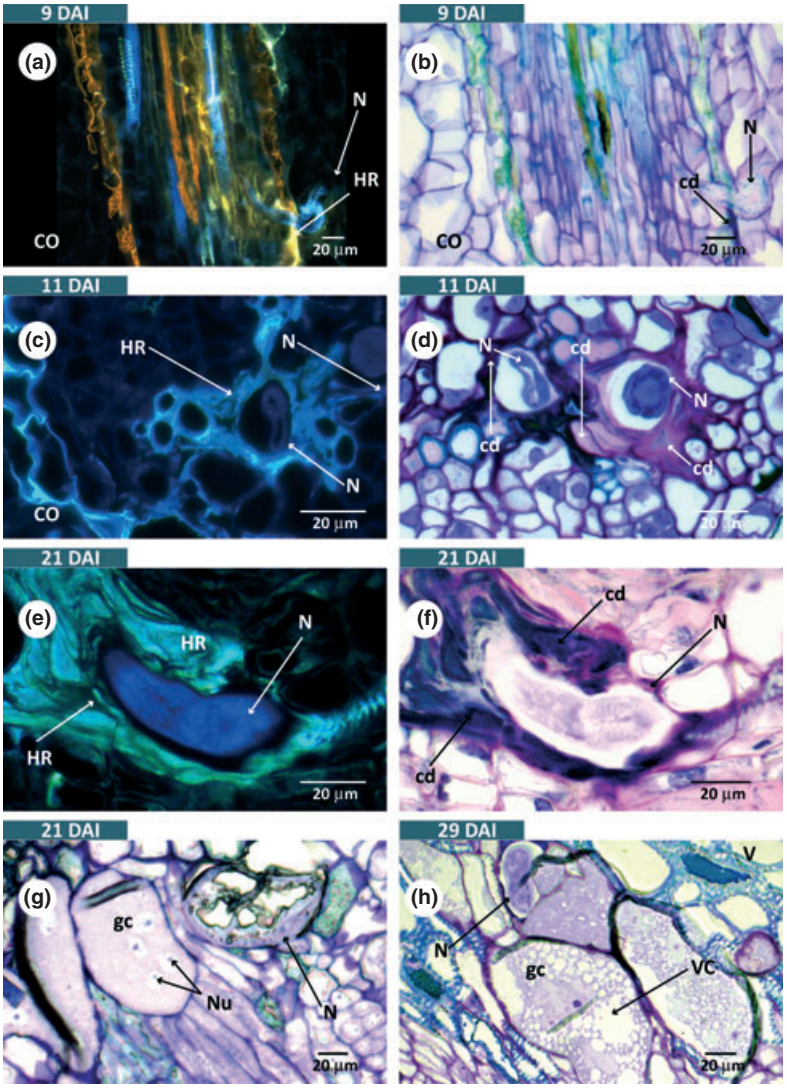

Figure 2 Resistance interaction. Roots of *Gossypium barbadense* accession CIR1348 infested with *Meloidogyne incognita* race 3. Bright field microscopical observations of sections stained with toluidine blue (b,d,f–h) and UV fluorescence microscopy of unstained sections (a,c,e). (c,d,f): J2/J3 present in the vascular cylinder. Host cells close to the nematode exhibit a bright yellow orange (a) or weak autofluorescence. A blue fluorescence is also seen in walls of vessels (a,c). (b,d,f): parenchyma cells close to juveniles exhibit a dark-stained material suggesting severe alteration of the cytoplasm. (g,h): giant cells showing retracted cytoplasm containing numerous small vacuoles; markedly deformed J3/J4 are seen in the vicinity of the altered giant cells. N = nematode, HR = hypersensitivity reaction indicating cell death (cd), CO = cortex, gc = giant cells, VC = vacuole, Nu = nucleus, V = vessel.

observed around the nematodes and giant cells. The nematodes were able to establish feeding sites in which the giant cells looked similar to those in susceptible roots before 14 dai (Fig. 3a). The first evident differences between the two genotypes (FM966 and TX-25) were observed at 14–18 dai when the giant cells adjacent to the nematode in resistant roots had some larger vacuoles that nearly took up the entire volume of the giant cells (Fig. 3b–e), whereas the giant cells in the susceptible roots had uniformly dense cytoplasm (Fig. 1e–g) with less vacuolation. Some nematodes before 14 dai were developing normally in the TX-25 accession based on observations of their size, shape and appearance of internal contents (Fig. 3a). However, the nematodes associated with resistant roots at 14 and 18 dai were arrested in development as they were slightly narrower than the nematodes in susceptible roots (Fig. 1f). At 18 dai, nematode mean body width was 10 μm in the resistant accession TX-25 (Fig. 3f), while it was 50 μm in the susceptible one (Fig. 1f). This is confirmed by giant cell deterioration at this stage, as most of the giant cells in the TX-25 roots appeared to be on the verge of collapse and devoid of any cytoplasm (Fig. 3f), while common cell walls between the giant cells were also thin present-

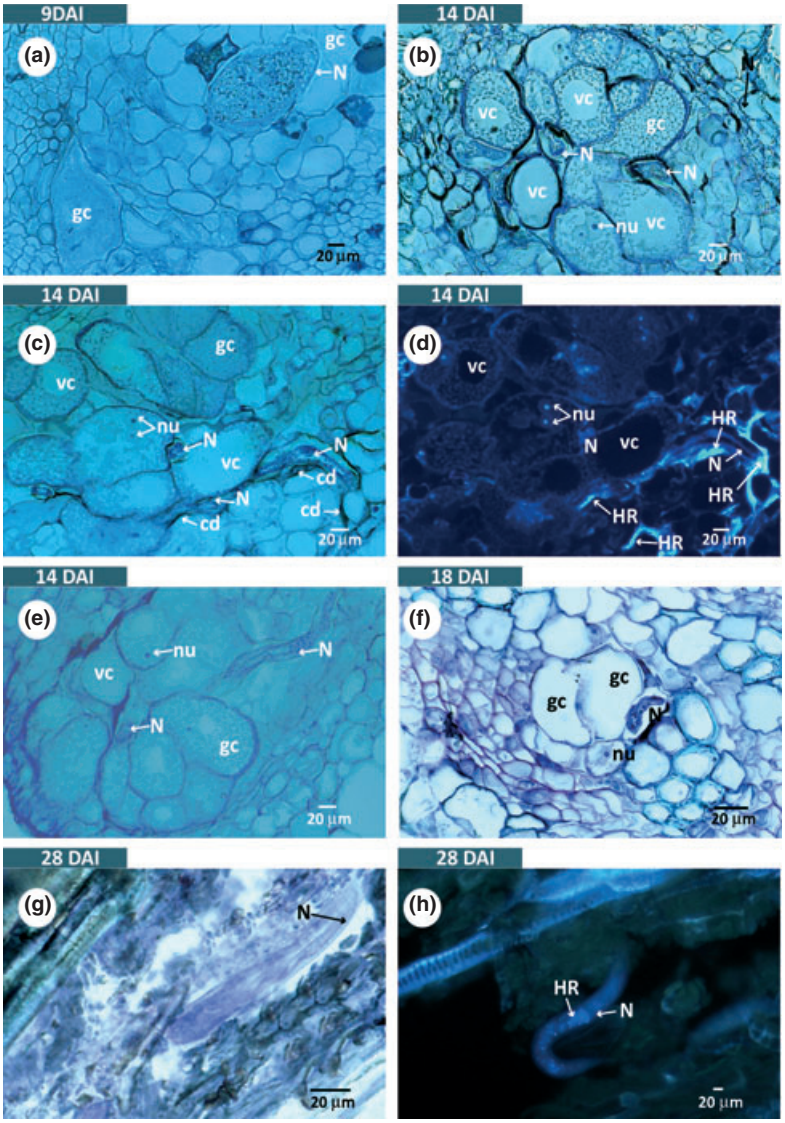

Figure 3 Resistance interaction. Roots of *Gossypium hirsutum* accession TX-25 infested with *Meloidogyne incognita* race 3. Bright field microscopical observations of sections stained with toluidine blue (a, b, c, e, f, g) and UV fluorescence microscopy of unstained sections (d, h). (a–f): J2/J3 present in the vascular cylinder. (b,c,e,f): giant cells showing retracted cytoplasm containing numerous vacuoles; markedly deformed J2/J3/J4 are seen in the vicinity of the altered giant cells. (d): host cells close to the nematode displaying blue fluorescence (HR) that is also seen in walls of vessels. (c,g): parenchyma cells close to juveniles exhibit a dark-stained material suggesting severe alteration of the cytoplasm. (h): juvenile with ultraviolet fluorescent material in the intestine. N = nematode, HR = hypersensitivity reaction indicating cell death (cd), gc = giant cells, vc = vacuole, nu = nucleus.

ing intercellular HR (Fig. 3c). In contrast, in susceptible roots, robust giant cell complexes with dense cytoplasm and thick cell walls were present (Fig. 1f,g). Deteriorated giant cells are not metabolically active enough to provide optimum nutrients for nematode development and reproduction. At 18 dai (Figs 1f & 3f) the differences in feeding sites between the two accessions (FM966 and TX-25) were clearly visible, showing empty giant cells in TX-25. At 28 dai the giant cell complexes in resistant roots of TX-25 had collapsed completely and root tissues were severely disrupted; only J2 after feeding (J2af) were observed (Fig. 3g,h) and none were observed to have advanced to a mature female stage. The prolonged presence of J2af can be explained by giant cell metabolic activity and/or increased production of phenolic compounds, providing suboptimal nutrition for nematode moulting and development up to 28 dai (Fig. 3b–h). The presence of putatively phenolic compounds (blue fluorescent material) is visible inside the intestine of J2s (Fig. 3h).

In susceptible roots at 28 dai, most of the nematodes had developed to mature females and their well-devel-

oped ovaries could be seen in the thin sections (Fig. 1h). Giant cell dimensions did not differ between infection sites in susceptible FM966 and resistant TX-25 roots until 14 dai. Giant cell dimensions measured after 14–18 dai were found to be larger in susceptible than in resistant roots. Although the TX-25 resistance reaction was delayed, the development of the majority of female nematodes was arrested in resistant roots such that they did not reach reproductive maturity. Thus, at 28 dai, residual galling was visible on resistant roots even though the giant cells were collapsed. External observations of the roots at 45 dai revealed large well-developed galls in susceptible roots, whereas the resistant TX-25 supported only some small galls. This confirmed the observations in screening essays: the nematode induced galls and produced egg matrices but failed to produce eggs (Tables 2 & 3). Probably the observed egg matrix contained a neglected quantity of eggs (data not shown).

Discussion

In the present study, 22 cotton accessions were tested for their ability to suppress RKN reproduction of a Brazilian isolate of *M. incognita* race 3. Ten of these had never been tested before and were chosen for possibly demonstrating some level of resistance to RKN (D. Dessauw, CIRAD, Montpellier, France, personal communication).

No general trend related to resistance or susceptibility was apparent within the three cotton species tested. Similarly, no relationship with geographical origin was seen, in agreement with previous studies (Robinson *et al.*, 2004).

Most of the accessions that had been previously tested showed responses in agreement with published results. Some discrepancies were noted between the present results and those obtained elsewhere. In the present assay, cultivar Auburn 56 was classified as resistant. This finding contrasts with previous studies where this same accession was classified as susceptible, moderately resistant, or intermediate between the two (Shepherd, 1983). Similarly, accession Wild Mexican Jack Jones (TX-2616) was resistant in the present study, while it is usually considered as moderately resistant, even though it has been described as root-knot resistant (Shepherd, 1974a) or tolerant (Shepherd, 1974b). Robinson & Percival (1997) tested the same accession, and in some – but not all – assays, it showed reproduction factors similar to that of the resistant control Auburn 623 RNR.

The most striking discrepancy between the results here and those previously published concerns the two wild *G. hirsutum* race marie-galante accessions AS0110 and AS0190. These accessions are wild entries from Haiti and Guadeloupe (Table 1). In their evaluation, Robinson *et al.* (2004) found both these accessions to be resistant, displaying a galling index equivalent to that of the resistant control Auburn 623 RNR and to that of TX-25. Variability in the aggressiveness of different cotton isolates of RKN has been documented (Elliot *et al.*, 1998; Zhou *et al.*, 2000). This variability could explain the slight discrepancies that were observed between some of the present results (Auburn 56, Wild Mexican Jack Jones) and those of previously published works. Discrepancies may also be accounted for by differences in the methodology used to evaluate resistance, which is sometimes based on galling index, at other times based on RF, or on both. Similarly, the number of days between inoculation and scoring varies amongst studies, and could also account for the differences. It is probable that all the above-mentioned differences account to a certain degree for the observed discrepancies.

Three highly resistant accessions were identified compared to the resistant control M-315 RNR: the wild Mexican *G. hirsutum* accessions Wild Mexican Jack Jones and TX-25 and the wild *G. barbadense* accession from Peru, CIR1348. No gall formation or very few galls were seen in Wild Mexican Jack Jones and CIR1348, while TX-25 exhibited a GI = 3, comparable to that of moderately resistant, susceptible or even highly susceptible accessions (Tables 2 & 3). This finding suggests the existence of different mechanisms of resistance among these accessions, and points to the fact that, although relatively easy to evaluate, GI is not the best measure to assess the level of resistance in cotton. Root galling has been positively correlated with egg production (Zhou *et al.*, 2000), and galling index has been proposed and used as a method for evaluating root-knot nematode resistance (Robinson *et al.*, 2004), but other works revealed that egg production was the best marker to develop cotton lines with high resistance to RKN (Carneiro *et al.*, 2005).

The highest level of resistance was found in the two wild accessions of *G. barbadense* from Peru (CIR1348 and CIR1343). South America, and in particular Peru, is considered the centre of origin and diversity of *G. barbadense* (Giband *et al.*, 2010). It is thus expected that a rich genetic variability is encountered in wild accessions from this region (Westengen *et al.*, 2005), including variability for resistance to RKN and other diseases or pests. This situation is similar to that of wild accessions and landraces of *G. hirsutum* from Mexico, the centre of origin of that species. Indeed, it includes the accessions Wild Mexican Jack Jones and TX-25, in which notable levels of resistance were identified (Robinson *et al.*, 2004).

The highly resistant *G. barbadense* accession CIR1348 and *G. hirsutum* TX-25 were chosen for further characterization of resistance mechanisms. Root samples of the susceptible control accession FM966 were used for comparative histopathological observations under visible bright and UV lights. J2 penetration was not affected in accessions CIR1348 and TX-25. Similar observations were made in the moderately resistant accession Clevewilt-6 (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). Pre-existing mechanisms which could impede nematode penetration seem to be apparently absent in

cotton, in contrast with a number of situations in which reduced penetration in resistant plants was reported (Niblack *et al*., 1986, Pegard *et al*., 2005; Proite *et al*., 2008). In accessions CIR1348 and TX-25, as in other RKN-resistant accessions of cotton, it appeared that resistance may result from post-penetration events associated with the blocking or delay of nematode development and reproduction.

In CIR1348 at least, two different mechanisms could be involved in the expression of resistance. One occurs at about 7 dai, which blocks J2s that have penetrated the roots, as observed for other RKN-resistant cotton accessions (Faske & Starr, 2009). In the resistant M-315 RNR, the number of J2s that had developed to reach the J3 and J4 stages was reduced in comparison to the susceptible control (Jenkins *et al*., 1995). Histological analysis showed that in accession CIR1348, this early defence reaction was concomitant to observation of an HR-like response. This response was shown to be involved in resistance against nematodes in a number of plant species, including coffee (Anthony *et al*., 2005), pepper (Pegard *et al*., 2005) and peanut (Proite *et al*., 2008). These HR-like areas in infected cortical cells displayed a yellow-orange autofluorescence under UV light, indicating the presence of phenols that could have a role in cotton defence (Nicholson & Hammerschmidt, 1992). Pegard *et al*. (2005) identified chlorogenic acid as the major phenolic compound present in root extracts of inoculated RKN-resistant pepper. Chlorogenic acid has been shown to be prejudicial to nematode survival, and its oxidation product significantly reduced their oxygen consumption (Macaron, 1975). The second defence mechanism in the CIR1348 accessions occurs at about 21 dai and further impedes the formation of adult females. Delay in nematode development has also been documented for a number of cotton RKN-resistant accessions. Jenkins *et al*. (1995) showed that at about 24 dai, the development to mature females was reduced in M-315 RNR compared to the susceptible control. Using the same accession Wubben *et al*. (2008) noted that by 21 dai, nematode development was arrested.

The few juveniles (J3/J4) that showed normal development in the CIR1348 accession at 21 dai were embedded in a dark blue toluidine-stained matrix which, under UV light, showed a light blue fluorescence. These J3/J4-stage juveniles, which showed an abnormal, deformed morphology, were observed in the vicinity of small feeding sites. Whereas in the susceptible control (FM966) giant cells were easily identified, those from the resistant accessions were much smaller in size, and were structurally altered, with degraded cytoplasm, a large number of small vacuoles, and thinner cell walls. Similar observations were made by Carneiro *et al*. (2005) in the RKN-resistant cotton breeding line IAC 96/414. The second resistance stage involved a mechanism that impedes the formation of functional feeding sites. Degenerated giant cells did not support the full development of egg-laying females, or only allowed females to lay a limited number of non-viable eggs

In primitive accessions of *G. hirsutum* (Faske & Starr, 2009) and in the M-315 RNR breeding line (Jenkins *et al*., 1995), a two-stage mechanism of resistance was also identified. Genetic analysis (Zhang *et al*., 2007) pointed out a 2-*R*-gene model for the inheritance of resistance. In their study, Jenkins *et al*. (1995) proposed that one gene acting at an earlier stage is responsible for the mechanism seen at 8 dai, while the second would explain the later (24 dai) phenomenon. Molecular mapping data (Gutiérrez *et al*., 2010) support these hypotheses, and revealed the occurrence of QTLs on chromosomes 11 and 14 to explain the resistance in cotton accessions (Auburn 634 RNR and M-240 RNR, respectively) which share the same source of resistance as M-315 RNR. The QTL on chromosome 11 is associated with reduced root galling index, while that on chromosome 14 is associated with reduced egg production (Gutiérrez *et al*., 2010).

Interestingly, J2 root penetration is not affected in *G. hirsutum* accession TX-25, and there was no evidence of early HR. In fact, the nematodes were able to initiate and maintain apparently healthy giant cells in resistant roots for about 2 weeks before visible signs of deterioration occurred, finally leading to giant cell collapse. The same was observed in resistant cowpea by Das *et al*. (2008). The fact that some giant cells and J2s after feeding exhibited typical HR or giant cell death associated with nematode death appears to be novel in cotton for RKN resistance. Delayed resistance response against RKN was reported in tobacco (Powell, 1962) where a late HR was also seen in developed giant cells. A common feature of pathogen-related HR is that it is preceded by loading of vacuoles with hydrolases and toxins and a calcium flux in the cytoplasm (Jones, 2001). A significant difference in vacuolation was seen between the resistant and susceptible cotton genotypes starting at 14 dai, and it is possible that the large vacuoles in resistant cotton TX-25 roots were filled with hydrolases and toxins that deprived the nematodes of nutrients and led to giant cell collapse. The delayed HR that occurs in TX-25 could explain the fact that this accession presented a GI = 3·0 (assay 1) or 3·5 (assay 2), while presenting a RF = 0·03 (assay 1) or 0·04 (assay 2). The small galls without egg masses probably represent a delayed biological cycle in the resistant TX-25 accession compared to FM966. Collapse of the giant cells at a later stage did not allow nematode development up to reproductive maturity and egg production.

Gossypium genetic resources constitute a rich source of resistance to RKN nematodes (Robinson *et al*., 2004), the study of which should be useful for cotton improvement. The Auburn 623 RNR breeding line (Shepherd, 1974a) that resulted from a cross involving the wild accession Wild Mexican Jack Jones still constitutes one of the most widely available resistant cotton accessions. However, the identification and characterization of novel sources of resistance is important to achieve effective and durable resistance to RKN in cotton. The present study contributed to this rationale objective. Despite similari-

ties and differences in resistance mechanisms found in the CIR1348 and TX-25 accessions and in the sources previously described, it is believed that they represent a novel source of a high level of resistance because they belong to a *Gossypium* background (*G. barbadense* and *G. hirsutum*) different from that of the presently available genetic material. Previous work (Starr *et al.*, 2010) has shown that primitive RKN-resistant *G. hirsutum* accessions appear to harbour different resistance genes. Classical and molecular genetics work aimed at further characterization of resistance found in the CIR1348 and TX-25 accessions will not only lead to determining the genetic and allelic relations between these sources and others, but also to developing molecular tools to effectively transfer the resistance to elite cotton germplasm.

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