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Marker-assisted selection in *Gossypium* spp. for *Meloidogyne incognita* resistance and histopathological characterization of a near immune line

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Abstract The root-knot nematode, *Meloidogyne incognita,* is one of the most important parasites that cause economic losses in the cotton crop. Plant genetic resistance is the most desirable strategy to control this pathogen. Sources of resistance in cotton have been known for several years but only a few resistant commercial varieties have been released. Cotton breeding lines were developed using marker-assisted selection in early generation plants to introgress root-knot resistance genes from two different sources: M-315 or CIR1348. Phenotyping was carried out in greenhouse conditions to validate the molecular markers associated with the resistance genes in the breeding lines and confirmed by genotyping. The markers targeting QTLs from M-315 resistance source were highly efficient in the selection of plants resistant to *M. incognita*, with all plants expressing a reproduction factor inferior to 0.08. CIR1348 resistance source markers were also very efficient in selecting resistance; however, some segregation events revealed the need for fine mapping of the resistance QTLs. To clarify the resistance mechanisms, present in the germplasm derived from the M-315 resistance source, the resistant line CNPA 17-26 B2RF (triple cross [BRS 368RF × M-315] × [BRS 430B2RF]) was chosen for histopathological characterization of plant-nematode interaction and compared with the susceptible FiberMax 966 (FM 966). The second-stage juveniles (J2) penetrated equally in both genotypes. In the histopathological study, a strong blue fluorescence was visualized in the tissues around the nematode (hypersensitivity reaction, HR), mainly at the beginning (from 2 to 6 DAI) in the cortex and central cylinder of the resistant plant, indicating accumulation of phenolic compounds in the roots. At 9 DAI, giant cells in the early stage of subdivision next to nematodes were observed in the central cylinder of the resistant plant, and phenolic compounds were also shown around the nematode. At 12–40 DAI these initial cells were completely degraded with the presence of phenolics involving the nematodes and initial giant cells. No fully developed giant cells or mature females were observed, only fourth-stage juveniles (J4s), and males were frequently visualized at 34 DAI. This resistance mechanism characterizes near-immunity, and so no enlarged females and no egg production were observed. In susceptible control, it

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was possible to visualize feeding sites well developed from 6 to 30 DAI. Females reached maturity at 26 DAI, and eggs were observed at 30 DAI. Our results suggested that the resistance (near-immunity) of the line CNPA 17-26 B2RF was related to early (2–12 DAI) defense responses that totally prevented nematode reproduction.

Keywords *Gossypium hirsutum* · *G. barbadense* · Hypersensitive response · Resistance genes · Root-knot nematode

Introduction

The genus *Gossypium* L. (collectively named cotton) comprises about 50 species (Wendel and Grover 2015) and is globally spread. Due to its natural diversification, different ancient human cultures on several continents have independently domesticated four species: two allopolyploids from the Americas, *Gossypium hirsutum* L. and *G. barbadense* L., and two diploids from Africa-Asia, *G. arboreum* L. and *G. herbaceum* L. (Wendel et al. 2009).

Gossypium hirsutum L. *latifolium* Hutch, referred to as Upland cotton, accounts for over 90% of world production (Jenkins 2003). *Gossypium barbadense* L., commonly known as Pima, Sea Island, Egyptian, or extra-long staple, represents approximately 5% of world fiber production (Wu et al. 2005). Upland cotton has been intensively cultivated in the Brazilian Cerrado biome since the early 1980's and, nowadays, more than 90% of cotton-growing areas are in this region (Silva Neto et al. 2016). The cotton supply chain contributed U$ 74 billion to the Gross Domestic Product (GDP) in 2017 and is responsible for generating over 1.3 million direct jobs (ABRAPA 2017). Recent genetic advances along with a better production system allow high fiber quality yields, ensuring international competitiveness of Brazilian cotton (Barroso et al. 2017). However, this tropical region is subject to high biotic stress pressure, and currently requires extensive pesticide inputs to achieve high levels of production. The root-knot nematode, *Meloidogyne incognita* (Kofoid and White 1919) Chitwood, 1949 is a serious pathogen and its importance has been increasing in cotton-growing regions (Galbieri and Asmus 2016).

Host resistance is the major focus of most crop disease management strategies. Genetic resistance is the most desirable strategy among farmers, as it promotes the reduction of pathogen populations, at the same time as allowing the cultivation of the crop of interest (Weaver 2015). However, few low-yielding cotton cultivars with high resistance to the root-knot nematode (RKN) are currently available in Brazil due to the difficulty of performing large-scale phenotyping to select resistant lines in cotton-breeding programs. This difficulty can be overcome using molecular markers linked to resistance QTLs (Suassuna et al. 2016). The implementation of marker-assisted selection (MAS) in routine procedures allows the selection of genotypes carrying desirable alleles and the advance of generations of crosses, therefore performing phenotyping with the nematode inoculation only in the most advanced stages of the program, with a limited number of lines (Yuksel et al. 2016).

Currently, two robust sources of resistance are available for cotton breeding programs in Brazil. The first is originated from Auburn 623 RNR breeding line, released to breeders in March 1970 (Shepherd 1974). Auburn 623 RNR is originated from a transgressive segregation derived from the crossing between two moderately resistant accessions, Clevewilt 6–3–5 and Wild Mexican Jack Jones (WMJJ). The high resistance found in Auburn 623 RNR has been transferred by a backcrossing method to give rise to the Auburn 634 RNR (Shepherd 1982) and several M-lines (Shepherd et al. 1996). These lines have been used by different cotton breeding programs, mainly M-120, M-240 and M-315 (Robinson 2008). Genetic resistance in M-lines appears to be oligogenically inherited, determined by at least two QTLs (McPherson et al. 2004) located on chromosomes 11 and 14 (Shen et al. 2006; Ynturi et al. 2006). The QTL (*qMi-C11*), originating from Clevewilt 6–3–5, has a dominant gene effect on the gall formation, and is in the interval CIR069-CIR316 on chromosome 11. However, QTL (*qMi-C14*), originating from WMJJ, has partial dominant gene effect, is associated with reduced egg production and is in the interval between BNL3545 and BNL3661 on chromosome 14 (Gutiérrez et al. 2010; Jenkins et al. 2012; Shen et al. 2010). An epistatic interaction between the two genes confers near-immunity resistance to RKN in the genotypes carrying both genes, which could not be explained

only by an additive effect of the two genes (He et al. 2014).

The second source of resistance, the accession CIR1348 (*Gossypium barbadense* L.) was recently described (Mota et al. 2012; Silva et al. 2014). The genetic mapping identified two major-effect QTLs on chromosome 11 and chromosome 15, which are responsible for a high level of resistance to the nematode (Gomes et al. 2016; Silva 2014). Although mapped in the same region on chromosome 11, QTL from CIR1348 (*G. barbadense*) has partially recessive oligogenic inheritance, a different inheritance mode from QTL from Auburn 623 RNR (*G. hirsutum*) and all derivative breeding lines (Gomes et al. 2016).

The associations between markers (CIR069, CIR316 and SHIN1425) and nematode resistance QTL on chromosome 11 and markers JESPR152 and NAU3254 on resistance QTL on chromosome 15 were confirmed (Gomes et al. 2016; Silva et al. 2014). The relationship of molecular markers with resistance genes has allowed the routinely use of MAS strategy in the EMBRAPA cotton breeding program. Several segregating populations were generated; MAS were used in early generations of plant selection, and a series of elite lines were obtained using M-315 or CIR1348 sources of resistance (Suassuna et al. 2019).

The mechanisms involved in the resistance response of *G. barbadense* CIR-1348 were elucidated through the histopathological characterization of the plant-nematode interaction with two post-infection mechanisms of resistance. The first mechanism of incompatibility occurred early after nematode penetration into cotton roots. This mechanism starts as a biochemical defense (hypersensitivity reaction—HR) that blocks the development of second-stage juveniles (J2) in other stages. The second mechanism prevents the development of J3/J4 into adult females and causes giant cell malformation and nematode deformities (Mota et al. 2012).

The ability to suppress nematode reproduction by Auburn 634 RNR and several M-lines derived from it has been known for a long time (Shepherd 1974). Resistance mechanisms have been investigated in M-315. Resistance genes do not alter J2 RKN penetration into cotton roots; however, M-315 resistance had a strong negative effect on nematode survival and reproduction, measured as number of eggs, egg-masses per plant, and eggs per egg-mass (Creech et al. 1995). Post-penetration development of RKN is also severely affected in M-315, appearing as a slower development of RKN: there are fewer developing third and fourth-stage juveniles in the initial 8 days after inoculation (DAI), and fewer developing to mature females at about 24 DAI (Jenkins et al. 1995). The *qMi-C11* affects gall formation and *qMi-C14* is associated with reduced egg production (Gutiérrez et al. 2010). Recently, it was confirmed that *qMi-C11* and *qMi-C14* act at different times and have different effects on the development of *M. incognita* and, therefore, have different modes of action (Silva et al. 2019). However, until now, an accurate histopathological characterization of a genotype with both *qMi-C11* and *qMi-C14* has never been performed.

Despite the beneficial effect of RKN resistance QTLs, the linkage drag of these resistance sources is pronounced, especially for low linter percentage and fiber quality traits. For breeding purposes, it is valuable to certify that resistance QTLs still function in a new genetic background after several backcrosses. In this study, we intend to confirm and validate the efficiency of SSR markers linked to different resistance genes sources to routinely use in early generation MAS. Additionally, we performed a detailed histological characterization of the resistance mechanism in a resistant line with *qMi-C11* and *qMi-C14* derived from M-315 showing that resistance acts throughout the cycle of the nematode, degrading the initial feeding sites, resulting in a very low nematode reproduction rate.

Materials and methods

Cotton germplasm

Lines derived from M-315 The segregating population was generated from a triple cross [(BRS 368RF × M-315) × BRS 430B2RF], where BRS 368RF and BRS 430B2RF are sources of resistance to cotton blue disease (CBD) and bacterial blight (BB), and M-315 to RKN. The population was advanced in bulk for three generations. F_4 plants were selected and genotyped for CBD, BB, and RKN resistance using SSR molecular marker DC20027 (Fang et al. 2010), CIR246 (Xiao et al. 2010), CIR316 and BNL3661 (Gutiérrez et al. 2010; Jenkins et al. 2012). All plants that amplified the homozygous band pattern associated with all

resistance genes (76 in total) were selected for progeny row tests. In the 2016–2017 season, 32 progenies were selected with good agronomic traits and were advanced to preliminary lines. From these, 19 lines were used in this study.

Lines derived from CIR1348 A cross and subsequent two backcrosses were performed using *G. barbadense* CIR-1348 as RKN resistance donor parent and *G. hirsutum* cultivar FM 966 as a recurrent parental. BC_2F_2 plants were genotyped using SSR markers CIR069, CIR316, SHIN1425, JESPR152 and NAU3254. All plants that amplified the homozygous band pattern associated with resistance QTLs were selected for progeny row tests in a net house. BC_2F_4 individual plants were selected in progeny rows, giving rise to the lines used in this study.

Divergent accessions Three divergent cotton germplasm accessions were included: a *G. hirsutum* var. *marie-galante* cultivar CNPA 5M, a *G. barbadense* line CNPA 2015-1800FL and a *G. hissutum* line previously described as a RKN-partially resistant, but not carrying any known SSR markers, CNPA GO 2002-2043/5. Details on accessions are in Table 1. These lines had not been previously evaluated, except CNPA GO 2002-2043/5. *Gossypium hirsutum* cv. FM 966 was used as a susceptible control and *G. hirsutum* line M-315 RNR was used as a resistant control.

Nematode inoculum

A pool of 15 *Meloidogyne incognita* populations collected in infected areas in Brazil was used for the phenotyping assay. Prior to inoculation, the populations were reproduced on tomato (*Solanum lycopersicum* L., cv. Santa Clara) for 3 months under greenhouse conditions. Eggs were extracted from infected roots using 0.5% NaOCl using a blender instead of manual shaking (Hussey and Barker 1973). For histopathological studies, freshly hatched second-stage juveniles (J2) were collected, using modified Baermann funnels; the population used was collected from a cotton farm in Bahia State, Brazil, and characterized as highly aggressive (Lopes et al. 2019).

RKN resistance in cotton germplasm

A total of 34 different accessions were tested. Eight individual plants of each germplasm, one per pot, were grown in pots (20 × 15 cm) filled with a mixture (1:1) of autoclaved soil and Bioplant® compost and maintained at 25–30 °C under greenhouse. Twenty-five days after seedling emergence, pots were inoculated with 10,000 eggs of *M. incognita* by pipetting nematode suspension around the stem base. Plants were arranged in a completely randomized design with eight replications. Plants were watered and fertilized as needed. Four months after inoculation, the root systems were rinsed under tap water and weighed. Roots were stained with Phloxine B and evaluated for gall and egg mass indexes, of which 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 and Sasser 1985). Eggs were extracted from roots in 1% NaOCl using the Hussey and Barker (1973) methodology, using a blender instead of manual agitation. The reproduction factor (RF) was calculated as RF = FP/IP, where FP = final nematode population and IP = initial nematode population (IP = 10,000). The average RF was transformed as $\log_{10}(x + 1)$, submitted to analysis of variance and the means grouped using Scott–Knott test ($P < 0.05$).

SSR genotyping

Young leaf tissue samples from all plants in the previous assay were used to extract total genomic DNA, using the CTAB method, and purification with chloroform: isoamyl alcohol. The DNA concentration was estimated by spectrophotometric reading, measuring the absorbance of the solution at wavelength 260 nm in a NanoDrop® 2000 Thermo Scientific spectrophotometer. All samples were genotyped with markers BNL3661 and CIR316 and lines derived from the source of resistance, CIR-1348, were also genotyped with markers CIR069, SHIN 1425, CIR316, JESPR152 and NAU3254. The PCR products were added to the GeneScan 500 ROX label and the plates were then placed in an ABI 3500XL automatic capillary sequencer. Analysis of the resulting peaks was done by the GeneMapper® program.

Histopathological analysis

Based on agronomic characteristics, phenotyping and genotyping, the line CNPA 17-26 B2RF was chosen for the histopathological analysis. Seedlings of CNPA

Table 1 Accessions of *Gossypium* spp. used in the study of marker assisted selection for resistance to *Meloidogyne incognita*

Cotton accessions	Species	Genealogy	Comments
FM 966	*G. hirsutum*	(DP90 × 75007-3) × (DP90 × Tamcot SP37H)	Commercial variety susceptible to RKN
CNPA 5M	*G. hirsutum* var. *marie-galante*	Recurrent selection in CNPA 3M	Obsolete cotton cultivar, supposedly tolerant do RKN
CNPA2015-1800FL	*G. barbadense*	Pima S-7 × Pima S-6	Allele 185/189 to BLN 3661 marker
CNPA GO 2002-2043/5	*G. hirsutum*	BRS Aroeira × (VAP 206 × Delta Opal)	Partial resistant line without known resistance alleles
CNPA T164-5	*G. barbadense* × *G. hirsutum*	([CIR1348 × FM 966] × FM 966) × FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPA T150-11	*G. barbadense* × *G. hirsutum*	([CIR1348 × FM 966] × FM 966) × FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPA T60-8	*G. barbadense* × *G. hirsutum*	([CIR1348 × FM 966] × FM 966) × FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPA T143-1	*G. barbadense* × *G. hirsutum*	([CIR1348 × FM 966] × FM 966) × FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPA T60-1	*G. barbadense* × *G. hirsutum*	([CIR1348 × FM 966] × FM 966) × FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPA T60-4	*G. barbadense* × *G. hirsutum*	([CIR1348 × FM 966] × FM 966) × FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPA T109-14	*G. barbadense* × *G. hirsutum*	([CIR1348 × FM 966] × FM 966) × FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPA T104-6	*G. barbadense* × *G. hirsutum*	([CIR1348 × FM 966] × FM 966) × FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPA T3-6	*G. barbadense* × *G. hirsutum*	([CIR1348 × FM 966] × FM 966) × FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPA T73-1	*G. barbadense* × *G. hirsutum*	([CIR1348 × FM 966] × FM 966) × FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPA 17-17 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-40 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-15 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-58 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-50 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-28 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-21 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-26 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-13 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-22 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-35 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-49 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-53 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-55 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-12 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-18 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-33 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-34 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
CNPA 17-56 B2RF	*G. hirsutum*	(BRS 368RF × M-315) × BRS 430 B2RF	Homozygous line to M-315 markers
M-315		USA—*G. hirsutum*	Breeding line with the RKN resistance QTLs *qMi-C11* and *qMi-C14*

17-26 B2RF and the susceptible check FM 966 were grown in plastic cups filled with washed and sterilized sand. 10-day-old cotton seedlings were inoculated with 10,000 J2s of *M. incognita* per plant. Two resistant and two susceptible seedlings per time point were carefully removed from the cups at 2, 4, 6, 9, 12, 15, 19, 23, 26, 30, 34 and 40 DAI and their roots were rinsed with tap water. Roots from three 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 (Axiophot Zeiss). Other roots were cut into small fragments of approximately 2 mm and fixed in 1% (1:1) solution of glutaraldehyde and 4% (v:v) formaldehyde in 100 mM phosphate buffer pH 7.2 for 24 h at 4 °C. Root fragment dehydration under agitation was carried out in an increasing ethanolic series of 10–100%, with intervals of 20 min between the exchanges. The root fragments were embedded in Technovit 7100® epoxy resin (Kulzer Friedrichsdorf, Germany) according to Pegard et al. (2005). The roots were cut with a Leica Ultracut UCT ultra-microtome in longitudinal and transversal slices of 3.5 µm thickness. Unstained root sections were mounted on glass slides and fluorescence was observed under UV excitation (Zeiss—Filter Set 01-488001-9901-000). Subsequently, the same sections were stained with 0.5% toluidine blue in 0.1 M sodium phosphate buffer, pH 5.5 (1 min at 60 °C) and observed under a light microscope. More than 6000 cuts were observed for the susceptible and resistant treatments.

Results

RKN resistance of cotton germplasm

Resistance was evaluated based on the criteria: gall index (GI), egg mass index (EMI), and reproduction factor (RF). The results of the phenotypic evaluation are shown in Table 2. The susceptible check, FM 966, exhibited high levels of gall and egg mass indexes, and the nematode had a high level of reproduction (RF average = 13). The accession CNPA 5M (*G. hirsutum* var. *marie-galante* (Watt) Hutch.) was the most susceptible (RF average = 35) and had the greatest variation around the mean (Table 2).

Along with phenotypic evaluation, complementary genotyping of each individual single plant was performed; genotyping results are synthetized in Table 3, and the expected allele sizes for each marker are in Table 4. In the genotyping assay, segregation was observed for the markers CIR 316 and BNL 3661 and different allele patterns appeared for the marker CIR 316, including alleles found in *G. barbadense*, *G. hirsutum* and one non-common allele, 192 (Table 3).

All genotypes derived from the CIR1348 resistance source, selected based on the markers JESPR152, SHIN 1425, and NAU 3254, significantly reduced nematode reproduction. From this group, the most resistant line, CNPA T73-1, had RF averages of less than 1; however, segregation was observed in lines CNPA T164-5, CNPA T150-11, and CNPA T60-8, in which one plant per treatment had RF values of 7.53, 9.97 and 4.7, respectively (Table 2).

The set of genotypes selected based on CIR 316 and BNL 3661 markers derived from M-315 were highly resistant (RF less than 0.01, Table 2), comparable to the resistant M-315 control.

Histopathology of the compatible interaction

Microscopic examination of acid fuchsin-stained roots and observation of toluidine blue-stained sections showed that a high number of second-stage juveniles (J2) were able to penetrate the root tip at 2 DAI (Fig. 1a). At 4 DAI some J2s reached the root central cylinder (CC), when it was possible to observe asymmetric initial giant cells, due to cellular disorder after nematode interaction (Fig. 1b). This cell disorder was visualized at 2, 4, 6 and 8 DAI, and the asymmetric cells in division caused root enlargement. At 6 DAI, oval-shaped, hypertrophied giant cells were observed (Fig. 1c) adjacent to well-developed J3 juveniles. The J3 changed into J4 at approximately 12–19 DAI (Fig. 1d). At 19 DAI, J4 were found in well-established feeding sites (Fig. 1e). Well-developed adult females were visualized at 30 DAI close to giant cells with thickened walls and some nuclei (Fig. 1f), and, at this time, the first egg masses were observed. At 40 DAI, empty giant cells next to females with many egg masses were visualized, and some re-infestation occurred as well.

Table 2 Mean phenotypic values of 34 cotton accessions with different levels of resistance to *Meloidogyne incognita*

Treat. no	Cotton accessions[a]	Reproduction factor		Gall index		Mass index	
		Mean[b]	Range	Mean	Range	Mean	Range
SC	FM 966	13.060 b	7.73–27.13	5.0	5–5	5.0	5–5
1	CNPA 5M	35.139 a	8.76–68.13	5.0	5–5	5.0	5–5
2	CNPA 2015-1800FL	8.216 c	4.271–14.61	5.0	5–5	5.0	5–5
3	CNPA GO 2002-2043/5	1.144 d	0.17–2.37	4.8	4–5	3.0	2–4
4	CNPA T164-5	1.637 d	0.33–7.53	4.3	4–5	2.4	0–5
5	CNPA T150-11	1.831 d	0.301–9.97	4.0	3–5	2.1	0–4
6	CNPA T60-8	1.230 d	0.40–4.70	3.6	3–5	2.4	1–4
7	CNPA T143-1	0.692 e	0.17–1.30	4.1	3–5	2.4	1–3
8	CNPA T60-1	0.519 e	0.17–1.37	3.4	3–4	2.5	2–3
9	CNPA T60-4	0.470 e	0.123–1.466	3.6	2–4	0.9	0–3
10	CNPA T109-14	0.276 f	0.168–0.568	3.8	3–5	1.9	0–3
11	CNPA T104-6	0.255 f	0.03–0.27	3.0	2–4	1.0	0–2
12	CNPA T3-6	0.28 f	0.03–1.13	2.0	1–3	1.1	0–3
13	CNPA T73-1	0.130 f	0.0–0.17	2.6	1–4	1.4	0–3
14	CNPA 17-17 B2RF	0.082 f	0.03–0.13	1.8	0–3	0.1	0–1
15	CNPA 17-40 B2RF	0.033 f	0.0–0.07	1.5	0–2	0.0	0–0
16	CNPA 17-15 B2RF	0.020 f	0.0–0.03	1.6	1–3	0.3	0–1
17	CNPA 17-58 B2RF	0.022 f	0.0–0.07	1.5	0–2	0.0	0–0
18	CNPA 17-50 B2RF	0.020 f	0.0–0.07	1.8	1–2	0.0	0–0
19	CNPA 17-28 B2RF	0.010 f	0.0–0.03	1.9	1–2	0.0	0–0
20	CNPA 17-21 B2RF	0.010 f	0.0–0.07	2.1	1–4	0.6	0–2
21	CNPA 17-26 B2RF	0.010 f	0.0–0.03	1.3	0–2	0.0	0–0
22	CNPA 17-13 B2RF	0.004 f	0.0–0.03	1.9	1–2	0.0	0–0
23	CNPA 17-22 B2RF	0.004 f	0.0–0.03	1.8	1–3	0.1	0–1
24	CNPA 17-35 B2RF	0.004 f	0.0–0.03	1.6	0–2	0.4	0–2
25	CNPA 17-49 B2RF	0.004 f	0.0–0.03	1.4	0–2	0.0	0–0
26	CNPA 17-53 B2RF	0.004 f	0.0–0.03	1.1	0–2	0.3	0–1
27	CNPA 17-55 B2RF	0.004 f	0.0–0.03	1.8	1–3	0.0	0–0
28	CNPA 17-12 B2RF	0.000 f	0.0–0.0	1.8	1–2	0.0	0–0
29	CNPA 17-18 B2RF	0.000 f	0.0–0.0	1.8	0–2	0.0	0–0
30	CNPA 17-33 B2RF	0.000 f	0.0–0.0	1.3	0–2	0.1	0–1
31	CNPA 17-34 B2RF	0.000 f	0.0–0.0	1.8	1–2	0.4	0–1
32	CNPA 17-56 B2RF	0.000 f	0.0–0.0	0.6	0–2	0.1	0–1
RC	M-315	0.020 f	0.0–0.07	0.6	0–2	0.0	0–0

SC susceptible check, *RC* resistant check

[a]Cotton accessions described in Table 1

[b]Mean values (from eight repetitions). Original values were transformed as log 10 (x + 1). Means followed by different letters within columns are significantly different ($P < 0.05$) according to Scott–Knot's test. Coefficient of variation (%) = 68.8

Histopathology of the incompatible interaction

Observations made using the (Byrd et al. 1983) methodology showed that the penetration of J2s occurred in similar number in susceptible FM 966 and resistant CNPA 17-26 B2RF accessions at 2–4 DAI close to the root tip. It was only after this migratory phase at 6 DAI that the J2s reached the central cylinder and the nematode became sedentary. From 12 to 30 DAI, J3s and J4s were visualized in central cylinder and some J4 females (Fig. 2f) and numerous males (Fig. 2g) were developed at 34 DAI, indicating that a certain number of juveniles turned into males and not into pear-shaped females. Mature root galls containing adult females and egg masses were not observed at 40 DAI.

In the resistant accession CNPA 17-26 B2RF, fluorescence microscopy using UV excitation of root

Table 3 Genotypes of SSR markers CIR 316, BNL 3361, CIR 069, SHIN 1425, JESPR152 and NAU 3254 in 34 cotton accessions

Resistance source		*G. hirsutum* M-315		*G. barbadense* CIR1348			
Chromosome		11	14	11		15	
Treat. no	Cotton accessions[a]/SSR marker	CIR 316	BNL 3361	CIR 069	SHIN 1425	JESPR152	NAU 3254
SC	FM 966	198/201	191/193/195	260	213	240	285
1	CNPA 5M	192/195/198/201/203	193/195				
2	CNPA2015-1800FL	192/203	185/191	264	221	174	277
3	CNPAGO 2002-2043/5	198/201	191/193/195	264	221	174	277
4	CNPA T164-5	201/203	191/195	264	221	240	277/285
5	CNPA T150-11	201/203	195	264	221	174	277
6	CNPA T60-8	201/203	191/195	264	221	174	277
7	CNPA T143-1	201/203	191/193/195	264	221	240	277/285
8	CNPA T60-1	201/203	191/193/195	264	221	174	277
9	CNPA T60-4	201/203	191/193/195	264	221	174	277
10	CNPA T109-14	201/203	193/195	264	221	174	277
11	CNPA T104-6	192/203	193/195	264	221	174	277
12	CNPA T3-6	192/203	191/193/195	264	221	174	277
13	CNPA T73-1	192/203	193/195	264	221	174	277
14	CNPA 17-17 B2RF	201/210	185/191				
15	CNPA 17-40 B2RF	201/210	185/191				
16	CNPA 17-15 B2RF	201/210	185/191				
17	CNPA 17-58 B2RF	201/210	185/191				
18	CNPA 17-50 B2RF	201/210	185/191				
19	CNPA 17-28 B2RF	201/210	185/191				
20	CNPA 17-21 B2RF	201/210	185/191				
21	CNPA 17-26 B2RF	201/210	185/191				
22	CNPA 17-13 B2RF	201/210	185/191				
23	CNPA 17-22 B2RF	201/210	185/191				
24	CNPA 17-35 B2RF	201/210	185/191				
25	CNPA 17-49 B2RF	201/210	185/191				
26	CNPA 17-53 B2RF	201/210	185/191				
27	CNPA 17-55 B2RF	201/210	185/191				
28	CNPA 17-12 B2RF	201/210	185/191				
29	CNPA 17-18 B2RF	201/210	185/191				
30	CNPA 17-33 B2RF	201/210	185/191				
31	CNPA 17-34 B2RF	201/210	185/191				
32	CNPA 17-56 B2RF	201/210	185/191				
RC	M315	201/210	185/191	260	213	240	291

Numbers in columns = Allele size (bp)

SC susceptible check, *RC* resistant check

[a]Cotton accessions described in Table 1

Table 4 Genotypes of SSR markers CIR 316, BNL 3361, CIR 069, SHIN 1425, JESPR152 and NAU 3254 in resistant and susceptible cotton germplasm to *Meloydogine incognita*

	G. hirsutum M-315		*G. barbadense* CIR1348			
Chromosome	C11	C14	C11		C15	
SSR marker	CIR 316	BNL 3661	CIR 069	SHIN 1425	JESPR152	NAU 3254
Resistant M-315	201/210	185/191	260	213	240	291
Resistant CIR1348	192/203	185/191	264	221	174	277
Susceptible FM 966	201/198	191/193	260	213	240	285

Numbers in columns = Allele size (bp)

sections harvested at 6 DAI showed a strong blue autofluorescence (UV) in several infection sites examined (Fig. 2a). Sections visualized under bright field microscopy after toluidine staining showed numerous cells in the central cylinder in the initial phase of division and dark blue staining, indicating necrosis and cell death at 9–12 DAI (Fig. 2b, d). A strong blue fluorescence was visualized, indicating accumulation of phenolic compounds, which is an indication of hypersensitivity reaction (HR) in the roots in the early stages (9–12 DAI) of infection (Fig. 2c, e). At 12 DAI a total degeneration of initial division cells was observed (Fig. 2d) with accumulation of phenolic compounds (Fig. 2e). At 23–40 DAI the root tissue adjacent to the nematode feeding site was completely degraded with the presence of cell death and HR: dark blue (Fig. 2h) and light or fluorescent blue (Fig. 2i). There was also pink staining, indicating unidentified polysaccharides (Fig. 2h). No giant cell or egg mass was observed, showing that the resistant line is practically immune.

Discussion

Selecting cotton nematode-resistant plants/lines based on field trials is a difficult task due to the irregular distribution of the pathogen in the soil and its interactions with other organisms, which could lead to false resistance selections. Nematode germplasm tests under a controlled environment are very efficient, since the plants are inoculated and evaluated individually, which, in turn, is very laborious and makes large-scale evaluation difficult. The recent knowledge on mapping and development of molecular markers associated with resistance genes and their application in breeding programs greatly facilitated the selection of resistant plants based only on genotypic analysis, leaving the phenotypic trials in greenhouse restricted to the final stages of the breeding program, evaluating advanced lines. Using these molecular tools, some progress in cotton nematode resistance has been achieved (McCarty et al. 2017; Suassuna et al. 2019).

Cotton germplasm is very diverse, and most of the useful gene pool remains unknown (Menezes et al. 2014). *Gossypium hirsutum* var. *marie-galante* is highly adapted to the Brazilian semi-arid environment, and its importance is mainly for small growers. Due to its wide adaptability, the germplasm CNPA 5M (treatment 1) was included in the set of cotton lines to be tested against the RKN. Nevertheless, CNPA 5M has shown high susceptibility to the parasitism of the nematode, and the genotyping test reveals the presence of both *G. hirsutum* and *G. barbadense* alleles, besides an additional unknown allele at CIR316 locus, implying a third yet unknown origin.

Pima cotton (*G. barbadense*) is known for its superior fiber quality (length, fineness, and strength) and is highly valued in the premium textile market. The 185/189 banding pattern was detected by BNL 3661 marker in eight *G. barbadense* accessions (de Carvalho et al. 2017). However, most of these accessions are highly susceptible to RKN. In a previous genotyping study, the accession CNPA 2015-1800 FL Pima (treatment 2) had the allele BNL 3661-185. In this study the presence of this allele (185/191 banding pattern) did not correlate with high levels of resistance in phenotyping. Although just few Pima cotton genotypes have been tested, all showing the allele BNL 3661-185 were RKN susceptible. A recombination event between allele BNL 3661-185 and the resistant gene may have occurred in *G.*

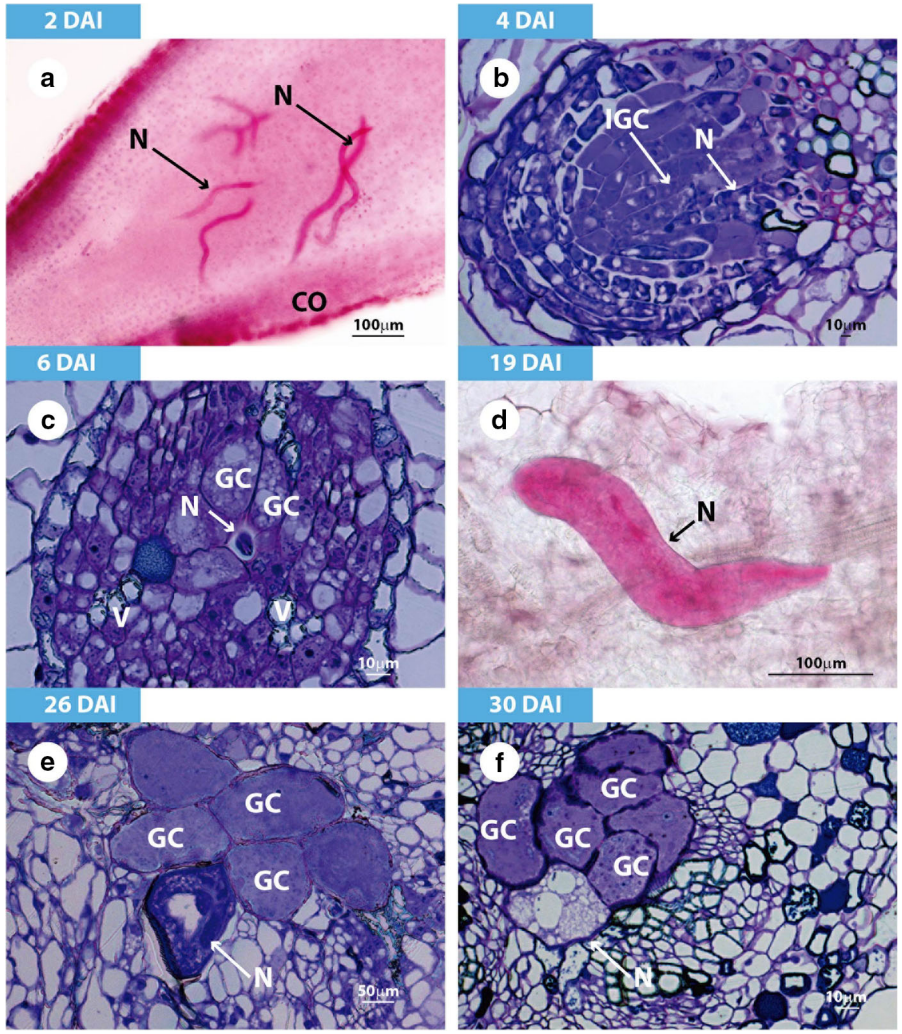

Fig. 1 Roots of *Gossypium hirsutum* cv FM 966 (susceptible control) infected with *Meloidogyne incognita*. **a**, **d** stained with acid fuchsin (af); **b**, **c**, **e**, **f** stained with toluidine blue. **a** nematode (J2) migrating towards the central cylinder at 2 days after inoculation (DAI); **b** initial giant cells close to nematodes in central cylinder at 4 DAI. **c**, **e**, **f** Nematodes and oval-shaped hypertrophied giant cells, with vacuoles or dense cytoplasm at 6, 26 and 30 DAI, respectively. **d** J4 stained (af) inside the central cylinder at 19 DAI. *N* nematode, *CO* cortex, *GC* giant cell, *V* vessel, *IGC* initial giant cell

barbadense or even the resistance gene does not exist in this species.

The resistance source Auburn 634 RNR, from which originated the M-315 line, has been studied extensively; its resistance is conferred by two QTLs, one of dominant effect and the other partially dominant or additive. One QTL was mapped on chromosome 11, called *qMi C11,* and it is associated with CIR316 SSR marker, while the second QTL was mapped on chromosome 14, called *qMi-C14* and associated with BNL 3661 marker (Gutiérrez et al. 2010; Jenkins et al. 2012; McPherson et al. 2004; Wang et al. 2006; Ynturi et al. 2006). Interval mapping results revealed that allele CIR316-201 exhibited a QTL peak located at 6.0 cM from *qMi-C11* and BNL 3661-185 at 10.05 cM from *qMi-C14* (Gutiérrez et al. 2010). However, based on our results, it can be inferred that all fragments involving both QTLs and the associated molecular markers were transferred to the breeding lines, since it was not possible to detect recombination events between the markers and QTLs. The 201/210 alleles from CIR316 marker associated with *qMi-C11*, as well as alleles 185/191 from BNL 3661 marker (*qMi-C14*), were found in all the lines

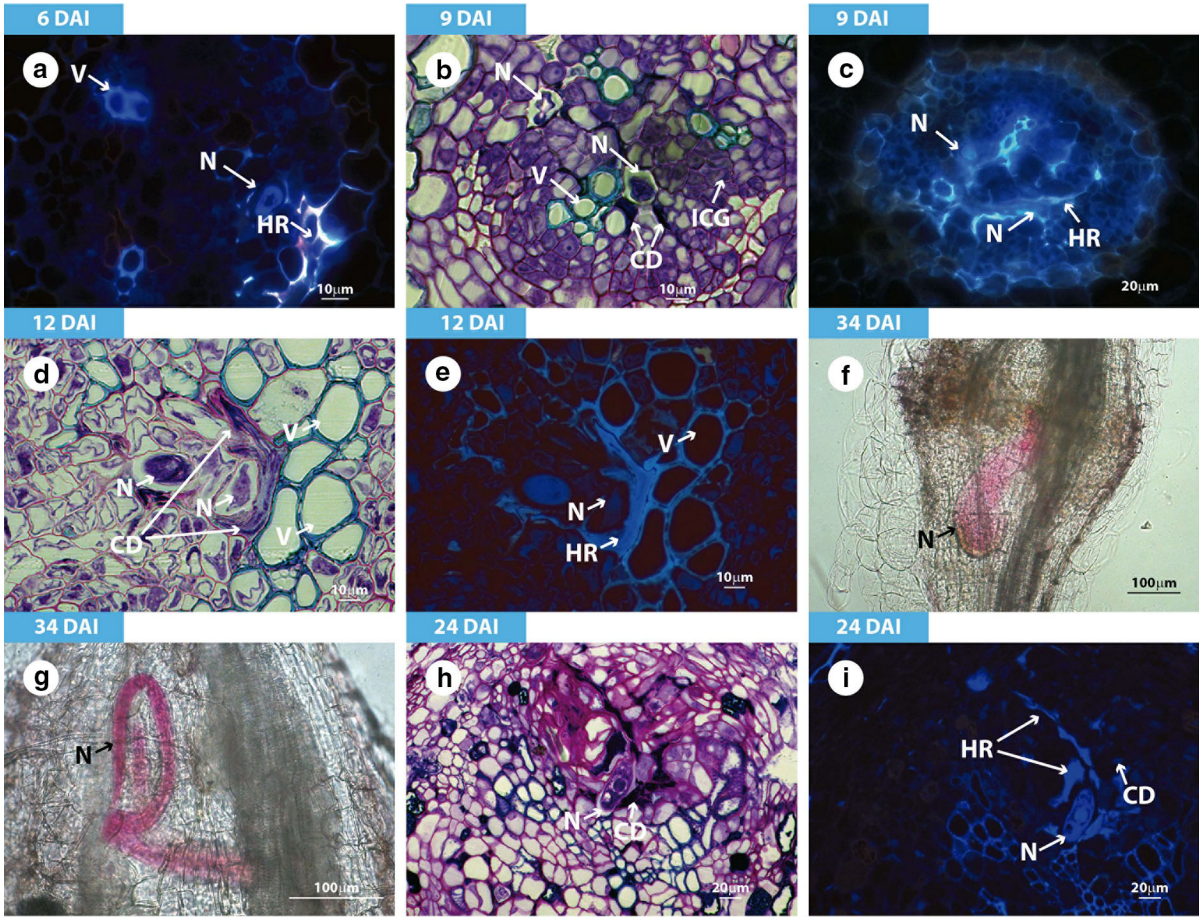

Fig. 2 Roots of *Gossypium hirsutum* accession CNPA 17-26 B2RF (resistant) infected with *Meloidogyne incognita*. **a**, **c**, **e** UV fluorescence observation. **b**, **d**, **h** Toluidine blue staining. **f**, **g** Acid fuchsin staining. **a** Strong fluorescence (hypersensitivity reaction, HR) in the root central cylinder at 2 days after inoculation (DAI). **b**, **c** Initial giant cells in division in central cylinder, cell death and HR at 9 DAI. **d**, **e** Disorganization of cells, cell death and strong fluorescence (HR) in central cylinder at 12 DAI. **f** Young female at 34 DAI. **g** Male at 34 DAI. **h**, **i** Cellular disorganization, cell death and autofluorescence (HR). *N* nematode, *CD* cell death, *V* vessel, *IGC* initial giant cell, *HR* hypersensitive reaction

originating from M-315 resistance source, and all plants selected based on these markers showed a very low RF (0.0–0.13). This result corroborates with previous studies using these same markers to select resistant plants from lines derived from Auburn 623 RNR (He et al. 2014; Jenkins et al. 2012; Ulloa et al. 2010). These findings confirm the efficiency of early generation MAS using SSR, targeting important QTLs in M-315 derived lines.

In 2014, Silva performed the genetic mapping of the new resistance source CIR1348 (*G. barbadense*) and detected one QTL on chromosome 11 flanked by the markers CIR069 and CIR316, and a second QTL on chromosome 15 flanked by the markers JESPR152 and NAU3254, with an average distance of 15.11 cM between adjacent markers. Studying an F_2 population that originated from the cross CIR1348 × FM 966, a ratio of 1/16 resistant individuals was found, which corresponds to a genetic resistance model regulated by two recessive genes, corroborating the results previously described (Silva 2014). It was demonstrated through histopathological characterization of plant-nematode interaction that the resistant genotype CIR1348 has two post-infective mechanisms of resistance (Mota et al. 2012). The source CIR1348 was the donor of resistance to lines CNPA T (treatments 4–13), markers CIR069, CIR316 and SHIN1425 (chromosome 11), and JESPR152, NAU3254

(chromosome 15), which were used for early generation by MAS in a population BC_2F_2. Although the makers linked to resistant alleles were correlated with low values of RF, GI and EMI, at least one plant in treatments 4, 5 and 6 (CNPA T164-5, CNPA T 150-11, CNPA T60-8) had high values for these variables. Possibly, the markers on chromosome 11 may have segregated in a block; however, recombination events may have occurred between the gene and the two markers. In this case, the putative gene is located on one side of both markers, which means that the markers would not be flanking the gene. Likewise, the same could have occurred with a plant in treatment 4 (CNPA T164-5); however, in treatment 4, the markers of the gene on chromosome 15 are in heterozygosis. Therefore, the molecular markers used for selecting resistant lines derived from CIR1348 clearly need to be fine-mapped, since several homozygous plants showed susceptible reactions, suggesting that some recombinants occurred between the marker and QTL.

The identification of a QTL on chromosome 15 by Silva (2014) suggests that this gene is a source of resistance different from those known to date. As the map obtained in that work is not totally saturated, it is interesting to add more markers to the regions of interest and thus to find markers that are as close as possible to the effective QTL. At this point, it is not possible to know if the locus of chromosome 11 found in CIR1348 is a gene other than that found in M-315. The study of a population obtained from the cross between CIR1348 and M-315 could clarify the relationship between the genes or alleles of these two sources of resistance.

Along with phenotyping and genotyping tests, the histological analysis of the inoculated roots also showed a strong difference in the nematode's ability to infect susceptible or resistant plant roots. The resistance to *M. incognita* detected in the *G. hirsutum* CNPA 17-26 B2RF line was investigated in a greenhouse test and through histopathological observations. The reproduction factor of *M. incognita* in roots of the resistant line was very low and characterized the near-immunity classification in this resistant genotype in comparison with the susceptible check FM 966. A strong response of the plant was observed, such as accumulation of phenolic compounds and cell death; in addition, the nematode was not able to develop females and reproduce in roots of the near immune line.

It is possible to categorize the mechanisms of resistance to root-knot nematodes in pre-infection and post-infection resistance (Anwar and McKenry 2002; Bendezu and Starr 2003). Pre-infection resistance is related to the failure of the nematode to penetrate the roots, which is due to the presence of toxic or antagonistic chemicals in root tissues (Bendezu and Starr 2003). In the post-infection resistance, nematodes can penetrate the roots, but fail to develop (Anwar and McKenry 2002). In this study, the number of J2 that penetrated the roots of susceptible and resistant accessions was similar and penetration occurred in both. The same event was observed in the genotype M-315 of *G. hirsutum* (Jenkins et al. 1995) and CIR1348 of *G. barbadense* (Mota et al. 2012). Pre-existing mechanisms which could prevent nematode penetration seem to be apparently absent in cotton, in contrast with several cases in which reduced penetration in resistant plants was reported (Pegard et al. 2005; Proite et al. 2008).

In resistant line CNPA 17-26 B2RF, two different mechanisms could be involved in the expression of resistance. One occurred at 2–6 DAI, which blocks J2s that have penetrated the roots, as observed in other RKN-resistant cotton accessions (Mota et al. 2012). Histological analysis showed that in line CNPA 17-26 B2RF, this early defense reaction was concomitant with observations of an HR-like response. The same mechanism was detected in CIR1348 (Mota et al. 2012). This response was shown to be involved in resistance to other RKNs in several plant species, including coffee (Lima et al. 2015), pepper (Pegard et al. 2005) and peanut (Proite et al. 2008). These HR-like areas in infected cortical or central cylinder cells displayed a blue autofluorescence, under UV light indicating the presence of phenolic compounds that could have a role in cotton defense (Nicholson and Hammerschmidt 1992). Pegard et al. (2005) identified chlorogenic acid as the major phenolic compound present in root extract of inoculated RKN-resistant pepper; they suggested that this acid is harmful to the nematode's survival and its oxidation product significantly reduced their oxygen consumption.

The second later defense mechanism in CNPA 17-26 B2RF line occurred at 9–12 DAI and prevented the formation of giant cells, the development of females and egg production (near immunity). This second hypersensitive response occurred in the central cylinder involving nematodes and initial giant cell

formation, and it was the most common mechanism manifesting as a post-infection event associated with rapid host cell death surrounding sites of initial infection by the nematodes. As a result, the pathogen is arrested and its development is completely inhibited (Williamson and Kumar 2006), and visible signs of deterioration occurred; leading to initial giant cells collapsing at 12–40 DAI.

In general, quasi-immunity (FR $\cong$ 0) was linked with early and late resistance mechanisms with initial HR and/or deterioration of the well-formed giant cells induced by *M. incognita* in resistant cotton (Mota et al. 2012) or resistant wild guava against *M. enterolobii* Yang and Eisenback, 1983 (Freitas et al. 2014).

Male sex conversion was visualized frequently in this study; it normally occurs when juveniles cannot establish appropriate feeding sites and nutritional conditions are not favorable to nematode development (Fassuliotis 1970; Pofu and Mashela 2011; Williamson and Hussey 1996). In this study, the presence of males can be explained by the fact of no formation of developed giant cells, with only initial ones that provided sub-optimal nutrition for female development at 26–30 DAI.

The results of this work clarified that cotton line CNPA 17-26 B2RF is an extremely efficient source of resistance because it prevents the formation of giant cells and females, totally compromising the reproduction of *M. incognita* race 3.

The line CNPA 17-26 B2RF has good agronomic traits, in addition to presenting the biotechnological event Bollgard II Roundup Ready Flex—B2RF; it can be launched as a cultivar and/or serve as a source of germplasm adapted to the tropical environment as a parental donor of resistance QTLs to RKN and other diseases of economic importance in Brazil.

It was recently launched in Brazil the cultivar IMA 5801 containing the resistance QTLs from the source M-315 and the same resistance mechanisms reported in this article. This cultivar is being planted in areas of high RKN infestation in the Brazilian Midwest (Galbieri et al. 2019).

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