UNIVERSIDADE DE BRASÍLIA
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE FITOPATOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM FITOPATOLOGIA

GENETIC AND PHYSIOLOGICAL VARIABILITY OF *Meloidogyne incognita* POPULATIONS FROM COTTON FARMS IN WESTERN BAHIA, AND EVALUATION OF RESISTANCE IN *Gossypium* spp. GENOTYPES

CARINA MARIANI LEITE LOPES

BRASÍLIA – DF

2019

CARINA MARIANI LEITE LOPES

GENETIC AND PHYSIOLOGICAL VARIABILITY OF *Meloidogyne incognita* POPULATIONS FROM COTTON FARMS IN WESTERN BAHIA, AND EVALUATION OF RESISTANCE IN *Gossypium* spp. GENOTYPES

Tese apresentada à Universidade de Brasília com requisito parcial para obtenção do título de Doutor em Fitopatologia pelo Programa de Pós-graduação em Fitopatologia.

Orientador

Prof. Juvenil Enrique Cares

Coorientadora

Dra. Regina Maria Dechechi Gomes Carneiro

BRASÍLIA – DF
2019

Trabalho realizado junto ao Departamento de Fitopatologia do Instituto de Ciências Biológicas da Universidade de Brasília, sob orientação do **Professor Juvenil Enrique Cares**, com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior **–** CAPES e Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq.

Genetic and physiological variability of *Meloidogyne incognita* populations from cotton farms in western Bahia, and evaluation of resistance in *Gossypium* spp. genotypes.

CARINA MARIANI LEITE LOPES

Tese Aprovada em 08 de maio de 2019 por:

_____

Prof. Cleber Furlanetto
Examinador Interno

_____

Dr. Camilo de Lelis Morello
Examinador Externo

_____

Dra. Patrícia Messenberg Guimarães
Examinador Externo

_____

Prof. Juvenil Enrique Cares
Orientador (Presidente)

BRASÍLIA – DF
2019

FICHA CATALOGRÁFICA

Lopes, Carina Mariani Leite.

Genetic and physiological variability of *Meloidogyne incognita* populations from cotton farms in western Bahia, and evaluation of resistance in *Gossypium* spp. genotypes./Carina Mariani Leite Lopes.

Brasília, 2019.

Número de páginas p.116: il.

Tese de Doutorado. Programa de Pós-graduação em Fitopatologia, Universidade de Brasília, Brasília, DF.

1. Genetic resistance, Cotton, Meloidogyne incognita

I. Universidade de Brasília. PPG/FIT.

II. Genetic and physiological variability of Meloidogyne incognita populations from cotton farms in western Bahia, and evaluation of resistance in Gossypium spp. genotypes.

Aos meus queridos avós, Lídia Menezes Lopes e José Antônio Teixeira Leite, que pelo enorme valor dado aos estudos mudaram o destino de meus pais e por tanto, o meu.

AGRADECIMENTOS

À Deus, por ser minha fonte inesgotável de fé, força e confiança,

Aos meus amados pais Sérgia Maria e José Cisino, meus irmãos Maurício, Rafael, Tiago e Samuel, Tia Stela e demais familiares por terem acreditado em mim em todos os momentos da minha vida me oferecendo seu apoio e amor incondicionais.

Ao meu querido João Bruno, por ter estado ao meu lado sempre me incentivando nesses últimos anos.

Ao meu tio Gustavo Mariani, que me acolheu e me ofereceu um lar confortável, durante toda minha estadia em Brasília.

Aos meus queridos orientadores Professor Juvenil Enrique Cares e Dra. Regina Maria Dechechi Gomes Carneiro, por terem confiado em mim e no meu trabalho, pela paciência e por todos os ensinamentos e ajuda durante a realização deste projeto.

À Universidade de Brasília – UnB/ Departamento de Fitopatologia pela oportunidade de realizar meu mestrado e doutorado.

Aos professores do departamento de fitopatologia da UnB com quem tive a oportunidade de aprender: Juvenil Cares, Cleber Furlanetto, Adalberto Café Filho, Carlos Uesugui, Helson do Vale, Rita Carvalho, Renato Resende, Carlos Lopes, Robert Miller, José Carmine Dianese, Marisa Ferreira, Danilo Pinho, Leonardo Boiteux, Alice Nagata, Luis Blum e especialmente ao querido amigo professor Claudio Lúcio, por terem dividido seus conhecimentos comigo e com meus colegas sempre com muita dedicação e paciência.

À CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior e CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico pela concessão das bolsas.

À EMBRAPA Recursos Genéticos e Biotecnologia, por ter acolhido minha pesquisa oferecendo toda estrutura necessária.

À Associação Baiana dos Produtores de Algodão – ABAPA pela confiança e valioso suporte financeiro.

À Associação de Agricultores e Irrigantes da Bahia por ter concedido o suporte logístico para a coleta das amostras.

Aos Doutores Fabiano José Perina e Nelson Dias Suassuna, pela parceria e amizade.

Às colegas do Laboratório de Nematologia da EMBRAPA – CENARGEN: Vanessa, Jessica, Marcilene, Daniela, Raycenne, Jorio, Caio e especialmente meu grande companheiro de trabalho Guilherme, por dividirem seus conhecimentos e almoços comigo.

E aos grandes amigos que conheci durante esses seis anos de mestrado e doutorado que estiveram sempre do meu lado torcendo por mim e fazendo com que essa experiência fosse uma das mais felizes da minha vida.

Meus sinceros agradecimentos!

SUMMARY

LIST OF TABLES 10
LIST OF FIGURES 11
RESUMO GERAL 12
GENERAL ABSTRACT 14
GENERAL INTRODUCTION 16
GENERAL OBJECTIVE 19
CHAPTER 1: LITERATURE REVIEW 20
Overview of cotton production 20
Cotton parasitic nematodes 22
The parasite *Meloidogyne incognita* 23
Biochemical and molecular identification of *Meloidogyne* spp. 28
Genetic diversity of the genus *Meloidogyne.* 31
Aggressiveness and virulence of *Meloidogyne incognita* 33
Molecular Markers Assisted Selection (MAS) 34
Simple Sequence Repeat (SSR) Markers 35
Resistance in cotton to *Meloidogyne incognita* 37
Mechanisms of resistance in cotton 40
REFERENCES 42
CHAPTER 2: Diversity of *Meloidogyne incognita* populations from cotton and aggressiveness to *Gossypium* spp. accessions 51
ABSTRACT 51
RESUMO 52
1. INTRODUCTION 53
2. MATERIALS AND METHODS 56
2.1 Characterization and identification of RKN species and races 56
2.2 Extraction of eggs and genomic DNA for genetic diversity and SCAR markers studies 58
2.3 Identification of *Meloidogyne* species by SCAR markers. 60
2.4 Characterization of genetic diversity of *M. incognita* by RAPD markers. 61
2.5 Characterization of genetic diversity of *M. incognita* by AFLP markers. 62
2.6 Phylogenetic analysis. 62
2.7. Aggressiveness/virulence of *M. incognita* populations on cotton accessions . 63
3. RESULTS 65
3.1 Characterization of nematode populations. 65

3.2 Genetic diversity of *Meloidogyne incognita*. 66
3.3 Aggressiveness/Virulence of *M. incognita* populations on cotton accessions. 68
4. DISCUSSION 71
REFERENCES 75
CHAPTER 3: Marker assisted selection for resistance in *Gossypium* genotypes to *Meloidoyne incognita*. 79
ABSTRACT 79
RESUMO 80
1. INTRODUCTION 80
2. MATERIALS AND METHODS 83
2.1 Cotton germplasm 83
2.2 Nematode inoculum 86
2.3 RKN resistance in cotton germplasm 86
2.4 SSR genotyping 87
3. RESULTS 87
4. DISCUSSION 92
REFERENCES 95
CHAPTER 4: Near Immunity resistance to *Meloidogyne incognita* characterize the line CNPA17-26 B2RF of *Gossypium hirsutum* 99
ABSTRACT 99
RESUMO 100
1. INTRODUCTION 101
2. MATERIALS AND METHODS 103
2.1 Plant material 103
2.2 Nematode population 103
2.3 Histopathology 104
3. RESULTS 105
3.1 Histopathology of the compatible interaction 105
3.2 Histopathology of the incompatible interaction 107
4. DISCUSSION 108
REFERENCES 111
CONCLUSÕES E CONSIDERAÇÕES FINAIS 115

LIST OF TABLES

Table 1. Differential hosts used to identify physiological races of *Meloidogyne incognita*, being (+) favorable host and (-) unfavorable host**……..27**

Table 2. List of *Meloidogyne incognita* populations and *M. enterolobii* (M. ent, outgroup) their origin (Brazilian municipalities and States), races and esterase phenotypes (Est)**.....57**

Table 3. Accessions of *Gossypium* spp. used in the study**…….....64**

Table 4. Percentage of polymorphisms observed in *Meloidogyne incognita* at populations level**.....68**

Table 5. Reproduction Factor (RF) of six populations of *Meloidogyne incognita* from Bahia State, in cotton accessions with different levels of resistance**.....70**

Table 6. Mean galling index (GI) and egg mass index (EMI) of six populations of *Meloidogyne incognita* from Bahia State on selected cotton accessions**..……..71**

Table 7. Eggs/(g) roots of six populations of *Meloidogyne incognita* from Bahia State, in cotton accessions with different levels of resistance**……..……....71**

Table 8. Correlation coefficients between the evaluation parameters for the cotton accessions tested**……..72**

Table 9. Accessions of *Gossypium* spp. used in the study of marker assisted selection of *Gossypium* spp. for resistance to *Meloidogyne incognita***…….……..……85**

Table 10. Mean phenotypic values of 34 accessions of cotton with different levels of resistance to *Meloidogyne incognita***……..…….……90**

Table 11. Alleles found to each marker in genotyping assay of *Gossypium* spp. lines**..91**

Table 12. Alleles expected to each marker in Marker Assisted Selection of *Gossypium* spp**……..……..92**

LIST OF FIGURES

Figure 1. The parasitic life cycle of *Meloidogyne incognita.* Scale bars, 50 μm. (Abad *et al*., 2009)**……24**

Figure 2. Isoenzyme esterase patterns for *Meloidogyne* species (Carneiro & Cofcewicz, 2008)**…….59**

Figure 3. a- Esterase phenotypes of *Meloidogyne incognita* (EST I2) 1 – 10: isolates from Bahia. P: *M. javanica* pattern (EST J3) included as reference. **b**- PCR amplification patterns of *Meloidogyne* spp. generated with specific SCAR primers inc-K14-F/R (Randig *et al*., 2002). 1 – 10: isolates from Bahia; 11 and 12: isolates from Paraná; 13: isolate from Mato Grosso do Sul; (I+, J+): positive controls, *M. incognita* and *M. javanica*, respectively. (-) DNA: negative control. M: 1 kb Plus DNA ladder (Invitrogen), bp: base pairs**.....67**

Figure 4. Genetic diversity of *Meloidogyne incognita* analyzed with primers RAPD Z4 **(a)** and AFLP 18 **(b)**. 1 – 10: isolates from Bahia; 11 and 12: isolates from Paraná; 13: isolate from Mato Grosso do Sul; 14: *M. enterolobii* (outgroup); (–): DNA negative control; M: 1 kb Plus DNA Ladder (Invitrogen); bp: base pairs**.....68**

Figure 5. Phylogenetic tree of *Meloidogyne incognita* populations from Bahia (1 – 10); Paraná (11 and 12) and Mato Grosso do Sul states (13) from RAPD and AFLP markers**……...……69**

Figure 6. Cotton roots after 4 reproduction cycles of *Meloidogyne incognita*. (a)-Susceptible check FM966 exhibiting several galls and egg masses; (b)-CNPA17-26B2RF line (treatment 21), roots without any gall or egg masses; (c)- CNPA T109-14 line (treatment 10), formation of few galls but none egg mass**……...……89**

Figure 7. Roots of *Gossypium hirsutum* cv FM 966 (susceptible control) infected with *Meloidogyne incognita.* A and D stained with acid fuchsin (af); B, C, E, and 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**……..107**

Figure 8. Roots of *Gossypium hirsutum* accession CNPA17-26B2RF (resistant) infected with *Meloidogyne incognita*. A, C, E – UV fluorescence observation. B, D, H – toluidine blue staining. F, G – acid fuchsin staining. A – 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**…….……109**

RESUMO GERAL

LOPES, Carina Mariani Leite. **Variabilidade genética e fisiológica de populações de *Meloidogyne incognita* em fazendas de algodão no oeste da Bahia e, avaliação da resistência de genótipos de *Gossypium* spp.** 2019. Tese (Doutorado em Fitopatologia) – Universidade de Brasília, Brasília, DF, Brasil.

O estado da Bahia é o segundo maior produtor de algodão do Brasil, sendo que esta cultura vem sofrendo severas perdas devido ao parasitismo do fitonematoide *Meloidogyne incognita*. Visando contribuir com informações relevantes para o uso da estratégia de resistência genética, objetivou-se estudar a diversidade genética, agressividade e virulência de populações desse nematoide oriundos da região oeste da Bahia, a qual responde por 96% da produção dessa fibra no estado, e avaliar a reação de linhagens de algodão a uma combinação de populações de *M. incognita*, bem como elucidar os mecanismos envolvidos na resistência de uma linhagem selecionada. Para isso, populações de *M. incognita* foram coletadas em 10 fazendas de algodão, multiplicadas em casa de vegetação e caracterizadas: molecularmente por marcadores espécie específicos tipo SCAR, bioquimicamente usando os fenótipos de esterase e fisiologicamente pela determinação das raças em hospedeiras específicas. Para os estudos de diversidade 53 primers RAPD e AFLP foram usados para acessar a variabilidade intra-específica e os resultados mostraram uma diversidade genética de 37,7%, pouco encontrada entre os isolados dessa espécie, sendo essa grande variabilidade devido à presença de uma população divergente (Pop. 8, Barreiras). No estudo da agressividade/ virulência nenhuma população se mostrou virulenta contra os genótipos de algodão moderadamente resistentes (Clevewilt-6,Wild Mexican Jack Jones e LA-887) e resistentes (CIR1348, e M-315 RNR), no entanto duas populações do município de Barreiras foram altamente agressivas à cultivar suscetível FM 966 (FR = 539 e 218), sendo uma delas, Pop. 8 a mais divergente geneticamente. As linhagens da

Embrapa com genes de resistência de duas fontes diferentes, M-315 e CIR1348, foram avaliadas por seleção assistida utilizando marcadores moleculares previamente desenvolvidos. Os marcadores moleculares associados aos genes de resistência foram validados através da fenotipagem com o nematoide em casa de vegetação e confirmados por genotipagem. Os marcadores originados da fonte de resistencia M-315 mostraram-se altamente eficientes na seleção de plantas resistentes a *M. incognita,* com 100% das plantas avaliadas expressando fator de reprodução inferior a 0,08. Já no caso dos marcadores da fonte de resistência CIR 1348, apesar de também serem eficientes na seleção de resistência, alguns eventos de segregação revelaram a necessidade de se buscar marcadores mais próximos aos QTLs de resistência. A linhagem resistente derivada de M-315, CNPA17-26B2RF selecionada para a caracterização histopatológica da interação planta-patógeno revelou um poderoso mecanismo de reação de hipersensibilidade que atua desde o início do ciclo de vida do nematoide com a liberação de compostos fenólicos no córtex e no cilindro central degradando os sítios de alimentação e impedindo que fêmeas maduras se desenvolvam, resultando em uma taxa de reprodução igual a zero. Esta linhagem foi selecionada por apresentar boas características agronômicas e por conter genes inseridosa por biotecnologia de resistência à insetos e herbicida (Bollgard 2 e Rundup Ready) além de marcadores para resistência a doença azul (virose), bacteriose e ao nematoide das galhas, podendo ser lançada como cultivar ou usada como doadora de resistência em programas de melhoramento.

Palavras-chave: Cotonicultura, nematoide das galhas, RAPD, AFLP, SSR, resistência genética, histopatologia.

GENERAL ABSTRACT

LOPES, Carina Mariani Leite. **Genetic and physiological variability of *Meloidogyne incognita* populations from cotton farms in western Bahia, and evaluation of resistance in *Gossypium* spp. genotypes.** 2019. Thesis (DSc. in Plant Pathology) – University of Brasília, Brasília, DF, Brazil.

The state of Bahia is the second largest producer of cotton in Brazil, but the crop suffers severe losses due to the parasitism of the plant-parasitic nematode *Meloidogyne incognita*. Aiming to contribute with relevant information for the use of plant genetic resistance , the objectives of this study were: to study the genetic diversity, aggressiveness and virulence of populations of *M. incognita* from western Bahia, which accounts for 96% of cotton fiber production in the state; to evaluate the resistance of elite cotton lines to a combination of populations of *M. incognita*; and to elucidate the mechanisms involved in the resistance of a selected line. For this, populations of *M. incognita* were collected in 10 cotton farms, multiplied in greenhouse and characterized: molecularly, by species specific SCAR markers; biochemically, using esterase phenotypes; and physiologically by the determination of races in different hosts. For the diversity studies 53 RAPD and AFLP primers were used to access the intra-specific variability in these *M. incognita* populations. The results showed a genetic diversity of 37.7%, rarely found among isolates of this species, being this great variability due to the presence of a divergent population (Pop. 8, from Barreiras). In the study of aggressiveness / virulence, no population was virulent to the moderately resistant (Clevewilt-6, Wild Mexican Jack Jones, and LA-887) and resistant cotton genotypes (CIR1348, and M-315 RNR), however two populations from Barreiras were highly aggressive to the susceptible cultivar FM 966 (Reproduction Factor - RF = 539 and 218).One of them, Pop. 8, being the most genetically divergent. Embrapa's cotton lines

with resistance genes from two different sources, M-315 and CIR1348, were evaluated by molecular markers assisted selection . The molecular markers associated to the resistance genes were validated by phenotyping with the nematode under greenhouse, and confirmed by genotyping. The markers originated from the resistance source M-315 were highly efficient in the selection of resistant plants against *M. incognita*, with 100% of the plants evaluated expressing RF of less than 0.08. The markers of the CIR 1348 resistance source were also efficient in the selection of resistance, however, some segregation events revealed the need to look for markers closer to resistance QTLs. The resistant line derived from M-315, CNPA 17-26 B2RF selected for the histopathological characterization of the plant-pathogen interaction has proved to harbor a powerful mechanism of hypersensitivity reaction that acts since the beginning of the nematode life cycle, with release of phenolic compounds in the cortex and the central cylinder degrading the feeding sites and preventing the development of mature females, resulting in a reproduction rate equal to zero. This lineage was selected due to its good agronomic characteristics and also harbor two biotechnological events (Bollgard 2 and Rundup Ready), and also containing genes for resistance to the blue virus disease, bacterial blight and to the root-knot nematode, which can be launched as cultivar or as donor of resistance in breeding programs.

Keywords: Cotton crop, root-knot nematode, RAPD, AFLP, SSR, genetic resistance, histopathology.

GENERAL INTRODUCTION

The species *Gossypium hirsutum* L. and *G. barbadense* L. are the main producers of natural fiber in the world. Brazil is the fifth largest producer of cotton, exclusively with *G. hirsutum*, concentrating its production in the Cerrado biome, mainly in four states: Mato Grosso (64.9%), Bahia (22.6%), Goiás (2.7%) and Mato Grosso do Sul (2.6%), which represent 93% of the growing area (CONAB, 2019). Bahia, the second largest cotton producer in Brazil and the largest producer in the Northeast region, concentrates 96% of its production in the west of the state.

Worldwide, various diseases and pests affect cotton productivity, including plant-parasitic nematodes, which are responsible for global agricultural losses amounting to an estimated $157 billion annually (Abad *et al*., 2009). In Brazil, the main nematodes that cause damages and losses to the cotton crop are *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949; *Rotylenchulus reniformis* Linford & Oliveira, 1940 and *Pratylenchus brachyurus* (Godfrey, 1929) Filipjev & Schuurmans-Stekhoven, 1941 (Inomoto, 2001). Among the parasitic nematode species of cotton plant, *M. incognita* has a wide distribution and survival capacity, besides having a wide range of host plants.

Most commercial cotton cultivars are susceptible to the root-knot nematode and only a few of them are moderately resistant to the pathogen in Brazil (Silva *et al*., 2014). Cultivar Auburn 623 RNR, resistant to *M. incognita* has been available for over 40 years (Shepherd, 1974a), from which the M-315 line has been derived, and widely used in studies as a source of resistance to the nematode. New sources of resistance are desirable for the development of cultivars with higher levels of resistance to the root-knot nematode and less likely to be supplanted by variants of the pathogen.

Currently, only one cultivar with high levels of nematode resistance is commercially available in Brazil, with the difficulty of performing large-scale phenotyping to select resistant lines in cotton breeding programs being one of the reasons. This difficulty can be overcomed by the routine use of molecular markers linked to resistance genes. The implementation of the use of molecular markers in routine, allows to select genotypes bearing desirable alleles and to advance generations, performing the nematode phenotyping only in the most advanced stages of the program, with limited number of lines.

Embrapa Cotton breading program has directed its efforts to develop cultivars with good agronomic characteristics combined with resistance to pathogens including the root-knot nematode (Suassuna *et al*., 2016). Using the marker assisted selection strategy (MAS) it was possible to develop lines with potential to meet these requirements, with the expression of the resistance confirmed by phenotyping.

The use of resistant cultivars in integrated management control requires the correct identification of nematode populations prevalent in the cotton growing areas (Ferraz *et al*., 2010), and the characterization of intraspecific genetic diversity among populations of *M. incognita*, originating from these areas.

Although, the occurrence of races is recognized in the species *M. incognita* (Taylor & Sasser, 1978), Moens *et al*. (2009) recommended that this terminology be discontinued, since a small variation among populations of the same species is measured and the range of hosts is very large. The analysis of avirulent and virulent populations to a given culture would be the most appropriate (Carneiro, 2015).

The objectives of this study were to characterize the intraspecific genetic diversity, aggressiveness and virulence of *M. incognita* populations from cotton growing areas in western Bahia, as well as the evaluation of the resistance level of

improved cotton genotypes inoculated with *M. incognita*. In addition, a study of the mechanisms involved in expression of resistance was conducted. The development and use of resistant cotton cultivars to the root-knot nematode could reduce quantitative and qualitative losses of fiber, besides representing an important management tactic in areas infested by the nematode in Brazil.

GENERAL OBJECTIVE

• To study the genetic and physiological variability of *M. incognita* populations collected in cotton farms in western Bahia, in order to evaluate the reaction of different cotton accessions in terms of resistance to *M. incognita*, and to clarify the resistance mechanisms involved in the control of this nematode.

SPECIFIC OBJECTIVES

• To determine the genetic variability present in populations of *M. incognita* collected in areas of cotton production in the state of Bahia using RAPD and AFLP molecular markers.

• To evaluate the aggressiveness / virulence of *M. incognita* populations collected in cotton farms of Bahia state, in hosts with of high and moderate resistance.

• To evaluate the resistance of elite cotton lines to a combination of *M. incognita* populations .

• To clarify the mechanisms involved in the resistance of cotton line CNPA17-26B2RF to *M. incognita*.

CHAPTER 1

LITERATURE REVIEW

Overview of cotton production

The cotton plant, *Gossypium hirsutum* L., produces a natural fiber of vegetal origin, with a length varying between 24 and 38 mm and is considered the most important of the textile fibers. In addition to fiber, cotton also produces oil and protein, which can be used as animal and human food supplements (Freire, 2014).

Cotton has been known to man since ancient times. The domestication of cotton occurred more than 4,000 years ago in southern Arabia and the earliest historical references to cotton are in the Manu Code, from the 7th century BC, considered to be the oldest legislation in India. The Incas in Peru and other ancient civilizations had already used cotton in 4,500 BC. The ancient writings, dating back to before the Christian Era, pointed out that the Indies were the main region of culture and that Egypt, Sudan, and all of Minor Asia already used cotton as a staple product (Beltrão *et al*., 2011).

In Brazil, when the Portuguese settlers arrived in 1500, cotton was already cultivated by the Indians, who converted it into yarns and fabrics for various purposes. At that time in Brazil, arboreal cotton was cultivated, with a perennial cycle, and in the 19th Century, herbaceous cotton, of annual cycle and shorter fiber, was introduced (Rodrigues & Bueno, 2004)

Nowadays, the tetraploid species *G. hirsutum* and *G. barbadense* L. are the two most cultivated species, accounting for approximately 95% of the cotton produced

worldwide. The diploid species *G. arboreum* L. and *G. herbaceum* L. are still cultivated in some areas of Asia (Freire, 2014).

The species *G. hirsutum*, whose original center of domestication is probably in Mexico, is the world's most widely grown cotton, known mainly as Upland cotton or herbaceous cotton, but also includes the American cotton varieties Acala and the cotton mocó (*G. hirsutum* race marie-galante) (Brubaker *et al*., 1999). Seven races of *G. hirsutum* are recognized: yucatan, punctatum, palmeri, latifolium, marie-galante, morrilli and richmondi (Hutchinson, 1951). Among these, punctatum, latifolium and marie-galante were dispersed, being considered the races from which the modern Upland cotton cultivars were derived (Lubbers & Chee, 2009).

Gossypium barbadense is a species from South America, whose center of domestication is Peru and Ecuador (Percy & Wendel, 1990; Westengen *et al*., 2005). It is constituted by two arboreal entities: race barbadense and race brasiliense. The latter composes the cotton known as the "bull-kidney" of the Amazon Basin (Brubaker, 1999). The elite cultivars of *G. barbadense* are represented by Sea Island, Creole, Egyptian, ELS, Indian and Pima (Percy, 2009).

Cotton is grown in more than 60 countries. The biggest producers are: India, China, United States, Brazil and Pakistan which together produce 77% of world production (USDA, 2018). In the 2018/2019, 2,413,000 tons of lint will be produced in Brazil. This production exceeds the previous cotton crop season in 16.9% and it is expected that Brazil will reach the mark of the world's second largest exporter of this fiber (ABRAPA, 2019; CONAB, 2019).

Brazil is the fourth largest producer of cotton exclusively with *G. hirsutum*, concentrating its production in the Cerrado biome, mainly in four states: Mato Grosso (64.9%), Bahia (22.6%), Goiás (2.7%) and Mato Grosso do Sul (2.6%), which represent

93% of the growing area. Although, cotton was grown in 14 Brazilian states in the last harvest, 87.4% of the planted area is concentrated in Mato Grosso and Bahia, which took place of São Paulo, Paraná and the Northeast states the main cotton producers at the beginning of cotton growing in Brazil (Rodrigues & Bueno, 2004; CONAB, 2019). Bahia, the second largest producer in Brazil, concentrates 96% of this fiber production in the western region of the State, where the largest investments in technologies are also found (CONAB, 2019).

In terms of yield, Brazil stands out with the highest index among world's largest cotton producers: 1,708 kg / ha in the 2017/2018 crop. China, with a yield of 1,257 kg / ha, has the second highest index, followed by the United States, with 984 kg / ha and India, with approximately 580 kg / ha (USDA, 2018; CONAB, 2019).

Cotton parasitic nematodes

A number of diseases and pests affect cotton yield in the world, and the most important nematode species are: the root-knot nematode, *Meloidogyne incognita*, which is found globally, and the reniform nematode *R. reniformis* Linford & Oliveira 1940, found in several tropical regions. The species *Hoplolaimus columbus* Sher, 1963 and *Belonolaimus longicaudatus* Rau, 1958, which may occur in harmful population densities in restricted geographic areas (Kirkpatrick & Rothrock, 2001), have no reports of occurrence in South America.

In Brazil, the main nematodes causing damages and losses to the cotton crop are *M. incognita*, *R. reniformis* and *Pratylenchus brachyurus* (Inomoto, 2001), and recently the nematode *Aphelenchoides besseyi* Christie, 1942 was associated to cotton plants exhibiting stunting, loss of floral buds, foliage distortion, and thickened nodes in Mato Grosso, and Bahia states (Perina *et al*., 2017; Favoreto *et al*., 2018).

Among the parasitic nematode species of cotton plant, *M. incognita* has a wide distribution and survival capacity, besides having a wide range of host plants. There are physiological races within the species (Veech & Starr, 1986); however, only the races 3 and 4 parasitize cotton, being race 3 the most widespread in commercial growing areas (Inomoto, 2001).

In the aerial part, a symptom frequently induced by the parasite is dwarfism and internerval chlorosis in the leaves "carijó", reduction of the volume of the root system and, as a consequence, reduction of the production and quality of the product (Silva & Santos, 1997). The damage caused by this nematode is higher in sandy soils with low fertility and when it is found in association with the cotton wilt causing agent, the fungus *Fusarium oxysporum* f. sp. *vasinfectum* Snyder & Hansen, forming the *Fusarium* x nematode complex (Asmus, 2004; Silva *et al*., 2004). Presence of *M. incognita* in high populations may turn cultivation unfeasible, with reports of abandonment of infested areas in the states of São Paulo, Paraná and Goiás (Galbieri *et al*., 2015).

The parasite *Meloidogyne incognita*

Biology and life cicle

The root-knot nematode (RKN) species of the genus *Meloidogyne* Goeldi, 1887 constitute a small part of the Phylum Nematoda, which comprises parasites of man, animals and plant, and species of free living in the soil, in fresh water and in the sea (Maggenti, 1981). The genus *Meloidogyne* is part of the Class Chromadorea, Order Rhabditida, Suborder Tylenchina, Infraorder Tylenchomorpha, Superfamily Tylenchoidea and Family Meloidogynidae (De Ley & Blaxter, 2002; Karssen & Moens, 2006).

The life cycle of the root-knot nematode is shown in Figure 1. The eggs of *Meloidogyne* are surrounded by a gelatinous matrix, which are usually deposited on the surface of the galls and sometimes they occur without the presence of galls. The embryonic development results in the juvenile of the first stage (J1) that undergoes an ecdisis still into the egg, giving origin to the juvenile of second stage (J2) which hatch from the egg by mechanical force exerted by its stylet, and also by the action of the chitinases produced in the esophageal glands and released through the stylet (Abad *et al*., 2009). The hatching of J2 *Meloidogyne* from the eggs is influenced by temperature and occurs in most species without the need of stimulating by the roots of plants (Ploeg & Maris, 1999).

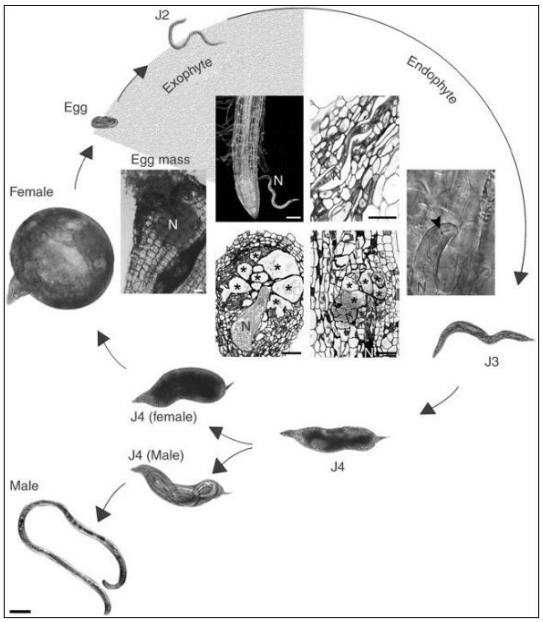

Figure 1. The parasitic life cycle of *Meloidogyne incognita.* Scale bars, 50 μm. (Abad *et al*. 2009).

When J2 hatches from egg mass, they infect neighboring galls or penetrate new roots. The J2 is attracted to the roots of plants and the location for infection depends on the perception of attractive gradients emanating from the roots. The infectious J2

accumulate in the region of cell elongation just behind the root tip, even in plants resistant to the RKN (Moens *et al*., 2009). They are also attracted to apical meristem points where the lateral roots emerge and places where other J2 penetrate. The nature of the stimuli released by the roots and perceived by the J2 is still unclear. Many organic and inorganic components excreted by the roots form gradients on the root surface into the soil and influence the nematodes. Carbon dioxide is often considered to be the most important factor of plant-nematode attraction (Karssen & Moens, 2006).

The J2 penetrate the rigid root walls by a combination of physical damage, through puncturing with the stylet and the action of cellulolytic and pectolytic enzymes. After root penetration the J2 migrate between the cells to the cortex in the region of cell differentiation. To circumvent the barrier formed by the endodermis, the J2 migrate toward the root tip, bypassing it until finding the apical meristematic region. They then travel through the vascular cylinder to the differentiation zone. Subsequently, they become sedentary in the cortical tissue (Taylor & Sasser, 1983; Karssen & Moens, 2006).

The J2 induces a feeding site, by converting parenchymatic cells into multinucleate giant cells where the nematode feeds. Then the J2 undergoes morphological changes, passing through three ecdysis to become juveniles of third and fourth stages and finally adults. Soon after the last ecdysis, the adult female starts feeding again, remaining inside the root for the rest of her life. During this post-embryonic development, the reproductive system develops and the functional gonads grow. Before the last ecdysis the piriform J4 male undergoes a metamorphosis in which the body lengthens, becoming a vermiform male (Eisenback & Triantaphyllou, 1991).

The life cycle of the root-knot nematode is greatly affected by temperature. Females produce eggs for about three months. Then the production stops, and they can

live a little longer. Males live weeks and J2s can live from a few days to months (Taylor & Sasser, 1983). In the case of *M. incognita* parasitizing cotton plants, at approximately 29 ºC, the first adult females appear from the 18thdays after penetration; the first eggs are found from the 26th to the 29st days (Mota *et al*., 2013)

Under normal conditions, almost all of the adults of *Meloidogyne incognita* are females. However, under unfavorable environmental conditions, with a high population of nematodes in the root or resistance of the host plant, juveniles that would develop into females, become males, this sexual reversion is one of the mechanisms of survival of these nematodes, as the population will reduce and the parasitism on the infected plant will be reduced, guaranteeing the survival of the formed females (Freitas *et al*., 2006).

Physiological races

Some *Meloidogyne* variants can only be separated from each other by their host preferences. These preferences are called biological or physiological races (Freitas *et al*., 2006).

The term race for the genus *Meloidogyne* does not have the same connotation of physiological race used in phytopathology. By definition, races are biotypes distinguished by their preference within a taxonomic group of host plants, in this case, the hosts are cultivars of the same species, differently from the usual separation of *Meloidogyne* spp., races which involves different species of plant (Moura, 1996).

The races of same species of *Meloidogyne* cannot be differentiated morphologically or genetically (Freitas *et al*., 2006). The identification of physiological races in *Meloidogyne* species is performed by test of differentiating hosts established at the North Carolina State University (Hartman & Sasser, 1985).

Meloidogyne incognita presents four physiological races (Eisenback, 1983). All races can reproduce in tomato (*Solanum lycopersicum* L.) cv. Rutgers; watermelon (*Citrullus vulgaris* Schrad) cv. Charleston Gray and pepper (*Capsicum annuum* L.) cv. Early California Wonder; however, their responses to the tobacco (*Nicotiana tabacum* L.) cv. NC 95, the cotton (*Gossypium hirsutum*) cv. Deltapine 61 and peanut (*Arachis hypogaea* L.) cv. Florunner vary with the race, as shown in Table 1.

Table 1. Differential hosts used to identify physiological races of *Meloidogyne incognita*, being (+) favorable host and (-) unfavorable host.

M. incognita	**Tobacco**	**Cotton**	**Pepper**	**Watermelon**	**Peanut**	**Tomato**
Race 1	-	-	+	+	+	+
Race 2	+	-	+	+	-	+
Race 3	-	+	+	+	-	+
Race 4	+	+	+	+	-	+

Source: Taylor & Sasser (1978)

The races 3 and 4 that parasitize cotton are present in practically all growing regions of the world, especially in areas with sandy soils and low fertility (Starr & Page, 1993). According to Fassuliotis (1985) the knowledge on races is important for the characterization of resistance in breeding programs. The identification of races in *Meloidogyne* spp. is essential to management strategies in infested areas, especially for crop rotation. For Lordello & Lordello (1996) the identification of races also allows knowing the distribution and the importance of each for local agriculture, as well as providing populations for the evaluation of genotypes and progenies in breeding programs.

However, the concept of race has never been universally accepted, in part because it measures only a small portion of the variation in potential capacity parasitic infection. Given the large numbers of hosts of many species, it is unlikely that the entire extent of variation is never adequately characterized (Moens *et al*., 2009), although recognition of

variation in the host range is important, these authors suggest that the formal recognition of physiological races based in positive reactions and / or in certain hosts to be discontinued.

Biochemical and molecular identification of *Meloidogyne* spp.

Esterase profiles

The use of isoenzymatic markers, such as esterase profiles and molecular markers based on DNA, have already allowed the correct identification of several species of *Meloidogyne* and proved to be reliable techniques (Blok & Powers, 2009).

Several biochemical studies with soluble proteins have been carried out in the last decades, and have shown that many species of the root-knot nematode can be differentiated by the enzymatic phenotypes obtained through polyacrylamide gel electrophoresis (Esbenshade & Triantaphyllou, 1985; Carneiro & Almeida, 2001).

The first researches using isoenzymes in the systematics of *Meloidogyne* spp., were developed by Dickson *et al*. (1971) and Hussey *et al*. (1972). At that time, about 30 enzymes were identified in several species of *Meloidogyne*. However, only the enzymes esterase (EST), malate dehydrogenase (MDH), superoxide dismutase (SOD) and glutamate oxaloacetate transaminase (GOT) have aroused interest from a taxonomic point of view. Later, Dalmasso & Bergé (1978) identified *Meloidogyne* spp. by extraction of proteins from individual females and separation of them through ultra-fine gel electrophoresis.

The technique of isoenzyme electrophoresis consists on the evaluation of the relative mobility (Rm) of polymorphic bands of isoenzymes. The enzyme mobility through polyacrylamide gel under electric current varies according to their electric charges and molecular weights, leading to the visualization of bands at different positions in the gel, which are specific for most species of *Meloidogyne*. The main

advantages of this technique include the recognition of *Meloidogyne* spp. even in mixed population, characterization of atypical populations, efficiency, reliability and speed (Carneiro *et al*., 2000; Blok & Powers, 2009).

Among the isoenzymes studied, esterases (ESTs) are the most used in the species identification of root-knot nematodes, with more than 40 phenotypes described (Blok & Powers, 2009). Other enzymes such as malate dehydrogenase, superoxide dismutase, and glutamate oxaloacetate transaminase are often used as aids in the characterization of previously identified species (Esbenshade & Triantaphyllou, 1985). One of the most relevant studies using isoenzymatic phenotypes to differentiate *Meloidogyne* spp. was published by Esbenshade & Triantaphyllou (1985), who reported esterase patterns for 16 *Meloidogyne* species, among them, phenotypes for *M. incognita*, *M. arenaria* (Neal, 1889) Chitwood, 1949, *M. javanica* (Treub, 1885) Chitwood, 1949 and *M. hapla* Chitwood, 1949.

Two esterase phenotypes I1 (Rm: 1.0) and I2 (Rm: 1.05, 1.1) are the most frequent to *M. incognita* and only one N1 malate dehydrogenase phenotype was characterized in all the studied populations (Carneiro *et al*., 2000; Castro *et al*., 2003). In recent work a third less common phenotype (S2N1) has been observed in some *M. incognita* populations, associated with coffee, banana, soybean and fig (Santos *et al*., 2012).

Despite their great utility, these markers cannot be used in studies of intraspecific variability (Arias *et al*., 2001). Intraspecific variability at the enzymatic level is generally very low because the enzymes are produced by the expression of highly conserved genes and represent only a very small fraction of the functional genome, whereas the non-coding regions are more abundant and undergo extensive evolutionary changes (McLain *et al*., 1987).

Scar markers

Although, still restricted to a few species, techniques involving molecular tools are excellent taxonomic methods for *Meloidogyne* spp. Molecular markers allow simple, accurate and rapid identification, although they do not allow detection of new or cryptic species, which are relatively frequent in the genus *Meloidogyne*, and are easily characterized by esterases (Blok & Powers, 2009).

In addition to the biochemical characterization, studies based on DNA analysis intensified since 1985, and recently, species-specific primers were developed, allowing a rapid identification of some species of *Meloidogyne* (Zijlstra, 2000; Randig *et al*., 2002; Mattos *et al*., 2019). The Polymerase Chain Reaction (PCR) technique has considerably advanced DNA analysis methods and led to the description of other classes of molecular markers, which associated with cloning and DNA sequencing techniques, have enabled a rapid accumulation of information on the structure of genomes (Ferreira & Grattapaglia, 1998).

A recent approach is the conversion of RAPD markers into SCAR, a term coined by Paran & Michelmore (1993) to define RAPD markers whose internal sequence has been determined, allowing longer, guanine and cytosine (GC) rich primers to be formed, of specific sequence. It is a very sensitive tool and allows the detection of species present in a mixture of populations in proportions equal to or less than 1% (Fourie *et al*., 2001; Randig *et al*., 2004). Among other advantages, it includes their use as physical reference points in the genome, for mapping, or as species-specific genetic markers, when they are associated with some genotype / phenotype of interest (Noir *et al*., 2003).

SCAR markers have already been developed to separate *M. incognita*, *M. javanica* and *M. arenaria*, (Zijlstra, 2000; Meng *et al*., 2004), *M. hapla* (Zijlstra *et al*., 2000) and quarantine species such as *M. chitwoodi* Golden, O'Bannon, Santo & Finley,

1980 and *M. fallax* Karssen, 1996. Randig *et al*. (2002) developed SCAR markers for the three main species parasites of coffee in Brazil: *M. incognita, M. exigua* Goeldi, 1887 and *M. paranaensis* Carneiro, Carneiro, Abrantes, Santos, & Almeida. 1996 and, Correa *et al*. (2013) included in this kit SCAR-coffee, two species occurring in coffee plantations in the Americas, *M. arabicida* López & Salazar, 1989 and *M. izalcoensis* Carneiro, Almeida, Gomes & Hernandez, 2005. SCAR markers were also developed for *M. enterolobii* Yang & Eisenback, 1983, the guava tree nematode, (Tigano *et al*., 2010), and *M. ethiopica* Whitehead, 1968 (Correa *et al*., 2014), and more recently for some species of the rice complex nematodes, *M. graminicola* Golden & Birchfield, 1965, *M. oryzae* Maas, Sanders & Dede, 1978, and *M. salasi* Lopez, 1984 (Mattos *et al*., 2019). When available, SCAR markers along with isoenzymes are interesting tools for diagnosis of *Meloidogyne* species.

Genetic diversity of the genus *Meloidogyne.*

The development of molecular techniques has opened new perspectives for studies on intraspecific variability of *Meloidogyne* spp. The use of PCR technique has brought significant advances in the implementation of new molecular markers associated with cloning techniques and DNA sequencing, allowing a rapid accumulation of information on genome structure (Ferreira & Grattapaglia, 1998).

PCR-based on RAPD (Randon Amplified Polymorfic DNA) technique is currently used in genetic studies and in the differentiation of *Meloidogyne* species from profiles generated with the aid of random primers (Cenis, 1993; Castagnone-Sereno *et al.*, 1994; Blok *et al*., 1997; Randig *et al*., 2002; Correa *et al*., 2013). In addition to using small amounts of genetic material, the technique does not require prior knowledge on the genome to be studied (Allan & Max, 2010). The main limitation of RAPD markers is the low content of genetic information per locus. Only one allele is detected,

while the other allelic variations are classified together as a null allele. Therefore, these markers behave as dominant and the data have binary nature (Ferreira & Grattapaglia, 1998).

Similarly to RAPD analysis, the Amplified Fragment Length Polymorphism (AFLP) technique (Semblat *et al*., 1998) allows the detection of specific and / or subspecific polymorphism in *Meloidogyne* spp. The advantage by this technique is the large number of fragments generated in a single gel (Ferreira & Grattapaglia, 1998). Another advantage is the great power of detection of genetic variability. It simultaneously exploits polymorphism of presence and absence of restriction sites and the occurrence or non-occurrence of amplification from arbitrary sequences, such as the RAPD assay, which characterizes a significant flexibility in obtaining polymorphic markers (Ferreira & Grattapaglia, 1998). The limitation of the technique, analogous to RAPD markers, is the low content of genetic information per locus. In addition, AFLP analysis involves a greater number of steps than RAPD (Ferreira & Grattapaglia, 1998).

Parthenogenetic species generally have low genetic variability. Parthenogenesis allows a fast reproduction, since there is no need to encounter the male with female as in amphimictic species. However, *Meloidogyne* species characterized by parthenogenetic reproduction such as *M. javanica*, *M. arenaria* and *M. incognita* have mechanisms that allows a rapid adaptation to unfavorable conditions, such as adaptation to resistant host plants (Trudgill & Blok, 2001). Randig *et al*. (2002) conducted a study on the diversity of Brazilian populations of *Meloidogyne* spp. and demonstrated a high degree of intraspecific variability in populations of *M. exigua*, *M. hapla* and *M. arenaria*, with 67.5%, 67.5% and 69.8% of polymorphic fragments, while *M. incognita* and *M. javanica* populations showed low intraspecific variability of 30% and 19%, respectively.

Studies on the genetic variability of *M. incognita* with the use of molecular markers have shown that the species has low genetic variability and the results have been consistent with the described isozyme phenotypes for the species. Although a few populations have been studied, the phenotypes I1N1 and I2NI of *M. incognita* were grouped with 100% similarity (Castagnone-Sereno *et al*., 1994; Blok *et al*., 1997; Randig *et al*., 2002; Carneiro *et al*., 2004; Cofcewicz *et al*., 2005; Santos *et al*., 2012). In the analysis of the intergenic region of mitochondrial DNA sequences, the phylogenetic tree showed that the isolate with the S2 / N1 profile is a variant of *M. incognita* with support of 89-79% bootstrap (Tigano *et al*., 2005).

Aggressiveness and virulence of *Meloidogyne incognita*

Aggressiveness reflects the ability of nematodes to reproduce in susceptible hosts, these being good or bad hosts. Veech & Starr (1986) defined aggressiveness, as a quantitative measure of the pathogen's ability to cause host damage, while virulence is the ability of the nematode to reproduce in hosts with resistance genes. In relation to virulence, there is the interaction of virulence genes with resistance genes that are respective in the parasite and in the host (Hussey & Janssen, 2002). Resistance breakdown events in natural populations of nematodes demonstrate the ability of the pathogen to develop mechanisms of adaptation to resistance genes, in case of continuous use of the same source of resistance or not (Castagnone-Sereno, 2002). Much of the information on virulence in *Meloidogyne* spp. is related with Mi resistance gene in tomato. In the 1950s it was observed a breakdown of resistance by *M. incognita*, *M. arenaria* and *M. javanica*, in field isolates, including in populations not previously exposed to the resistant cultivar, which were named as B-races. (Riggs & Winstead, 1959). Since *M. incognita*, *M. arenaria* and *M. javanica* are species that reproduce mandatorily by mitotic parthenogenesis, other mechanisms of genetic recombination

must be responsible for the increase of virulence in these populations (Hussey & Janssen, 2002).

The selection of virulent populations of *M. incognita* after successive plantings of resistant cotton occurred in California (Ogallo *et al*., 1997) and Texas (Zhou *et al*., 2000). In these cases, nematode isolates, with higher levels of reproduction in the NemX resistant cultivar were found in fields previously planted with this source of resistance. The identification of multiple sources of resistance, which can be used in gene rotation or pyramiding, thus becomes even more important.

Molecular Markers Assisted Selection (MAS)

At the beginning of plant breeding programs, the selection procedure of genotypes with desirable traits was performed only on the basis of the phenotypic information of individuals. But the emergence of DNA marker techniques with the ability to detect additional genetic variation has brought advances for genetic plant breeding. The main collaboration of this technique is to make it possible to analyze the genotype of an individual by means of a molecular marker, without the need of the occurrence of phenotypic expression, therefore excluding influence of the environment (Mohan *et al*., 1997).

The Marker Assisted Selection (MAS) has some advantages in relation to phenotypic selection. Examples include gene pyramiding, which seeks to concentrate different characteristics of interest in a single genotype (for example, resistance to different pathogens), with the use of MAS there is a reduction in the time needed to obtain this genotype. MAS is also used to select characters in which phenotypic evaluation presents a high cost, or requires specific environments (water deficit, inoculation of pathogens), when they require destruction of the plant and when the character of interest only manifests in advanced stages of plant development. Another

advantage over phenotypic selection is that MAS can be performed in the initial stages, significantly reducing the time required for a selection cycle (Xu & Crouch, 2008).

One of the earliest studies on marker-assisted selection was done by Stuber *et al*. (1982), with the use of eight isoenzymatic loci in a maize breeding population. The breeding program aimed at soybean resistance to the cyst nematode has been aided by MAS (Concebido *et al*., 1996; Schuster *et al*., 2001). There are several types of molecular markers available for genetic study, being the most used are izoenzymes, RFLP, RAPD, AFLP, microsatellites (SSR) and more recently SNPs (Mohan *et al*., 1997, Mamadov *et al*., 2012).The era of molecular markers in genetics began in the 1950s, with the discovery that allelic forms of enzymes could be separated by electrophoresis, As early as the 1980s, DNA-based markers, such as RFLP and RAPD, were used. Molecular markers, in comparison with morphological markers, have the advantages of being phenotypically neutral, polymorphic, abundant, and some of them have codominant inheritance (Tanksley, 1993).

Simple Sequence Repeat (SSR) Markers

Microsatellite markers were first developed in humans and soon received the attention of plant breeders and geneticists, as several studies have shown that microsatellites are widely distributed in the genomes of species (Brunel, 1994; Litt & Luty, 1989). In eukaryotic genomes, these simple sequences are very common, randomly distributed, besides being highly polymorphic genetic loci. In drosophila, 30% of the genome is formed by this type of sequence; in rice, 50%; and in tobacco it reaches 70% (Ferreira & Grattapaglia, 1995; Lewin, 2001). Molecular markers SSRs have been widely used for assisted selection of genotypes in breeding programs.

Microsatellite or SSR markers are sets of one to six nucleotide sequences ('motif') that repeat ('tandem') at various points in the eukaryotic genome (Litt & Luty, 1989).

According to Brondani *et al*. (1998), microsatellites are very attractive to plant breeders because they combine several advantages, such as: 1) codominant and multi-allelic nature; 2) because they are highly polymorphic, they allow accurate discrimination even of highly related individuals; 3) are abundant and uniformly dispersed throughout the plant genome; 4) can be efficiently analyzed by PCR; 5) marker information, based on primer sequences, can be easily published and exchanged between laboratories, enhancing cooperative research and development efforts.

The DNA sequences flanking the SSRs are generally conserved within the same species, allowing the selection of specific primers that amplify via PCR, fragments containing the replicate DNA in all genotypes. When the microsatellites are individually amplified, using the primer pair complementary to the sequences flanking them, they have size polymorphism. This variation in size of the PCR products is a consequence of the occurrence of different numbers of repetitive units within the microsatellite structure. Thus, several alleles can be determined for a given population. Homozygous individuals have the same number of repeats on homologous chromosomes, while heterozygous individuals have different numbers of replicates in the homologues chromosomes. Therefore, the locus is defined by the pair of primers and the various alleles, by the size of the amplified bands (Mohan *et al*., 1997; Ashkenazi *et al*., 2001; Mamadov *et al*., 2012)

The difference in size between the amplified DNA fragments can be detected by electrophoresis in polyacrylamide gels or capillary system with semi-automated detection via fluorescence spectral laser in an automated DNA sequencer (Mamadov *et al*., 2012).

SSRs are very common and randomly distributed throughout the genome, allowing complete coverage of the chromosomes of a given species. In addition to their

use for mapping genomes, microsatellites are ideal for identifying and discriminating genotypes and for population genetics studies. In cotton, several groups of researchers are developing genetic markers and SSR-based maps (Zhang *et al*., 2008, Yu *et al*., 2011; Fang & Yu, 2012).

Resistance in cotton to *Meloidogyne incognita*

There are few cotton cultivars resistant to the root-knot nematode with high potential yield and good fiber quality. In the USA, three major sources of resistant germplasm are recognized in Upland cotton (Robinson *et al*., 2001).

The first and most important source of resistance is from Auburn 623 RNR, which originated from transgressive segregation in a cross between two moderately resistant parents, Clevewilt 6 and 'Mexico Wild' Jack Jones, TX-2516 (Shepherd, 1974a, b). The resistance was subsequently transferred to several agronomically adapted cultivars through backcrossing, resulting in the release of the M-line series including lines such as 'M-120 RNR', 'M-135 RNR', 'M-155 RNR' and 'M-315 RNR', which greatly improved the agronomic qualities while retaining the almost-immune level of resistance of Auburn 623 RNR. Clevewilt 6 was also used to develop the root-knot nematode resistant line LA434-RKR, which represents the second source of resistance, and third, there is resistance found in a commercial Acala cultivar, Acala NemX (Ogallo *et al*.,1997). Resistance in Acala NemX cotton is conferred by the recessive gene rkn1 (locus Mi2h-C11) on chromosome 11. The original source of resistance in Acala NemX is uncertain, but the donor parent N6072 is thought to have been derived from crossing a *G. barbadense* L. genotype and the Acala line 1-2302 (Robinson *et al*., 2001).

Evidence of a two-gene system was obtained from crosses with the M-series lines (McPherson, 2004; Zhou *et al*., 1999). In an earlier study, using the bulk segregant approach, Shen *et al*. (2006) identified a major QTL on Chromosome 11 in the resistant

line M120 of the Auburn 623 RNR source using an interspecific population derived from crossing the resistant line with the susceptible *G. barbadense* cultivar "Pima S-6". This QTL, which influences root galling and was inherited from the Clevewilt 6 parent (Shen *et al*., 2006) was independently validated (Ynturi *et al*., 2006; Niu *et al*., 2007; Gutiérrez *et al*., 2010) and has been fine mapped to a region of 3.6 cM interval (Shen *et al*., 2010). Working with a different M-series resistant line of the Auburn 634RNR source, Gutiérrez *et al*. (2010) reported a second QTL located on Chromosome 14. Unlike the QTL on Chromosome 11, this QTL appears to largely influence RKN egg production and was inherited from the Mexico Wild Jack Jones parent (Gutiérrez *et al*., 2010).

A new source of high level resistance to the nematode *M. incognita*, the access CIR1348 of *G. barbadense* was identified in Brazil (Mota *et al*., 2013; Silva *et al*., 2014). In contrast to the Auburn 623 RNR line, the high resistance of the CIR1348 accession has partially recessive oligogenic inheritance. In the CIR1348 accession, two QTLs were identified, one on chromosome 11 and another on chromosome 15, which would be responsible for the high level of nematode resistance (Silva *et al*., 2014; Gomez *et al*., 2016). The association between markers (CIR069, CIR316 and SHIN1425) and QTL of nematode resistance on chromosome 11 and markers (JESPR152 and NAU3254) on resistance QTL on chromosome 15 were confirmed (Silva *et al*., 2014; Gomez *et al*., 2016).

Most commercial cotton cultivars are susceptible to the root-knot nematode and only a few of them are resistant to the pathogen in Brazil (Silva *et al*., 2014). Carneiro *et al*. (2005) evaluated six cotton genotypes available from the Instituto Agronômico de Campinas (IAC) selected under field conditions for resistance to *M. incognita* race 3. Among the evaluated genotypes, IAC 20-233 and IAC 20 RR-98/409 were considered

moderately resistant, whereas IAC 96/414 showed resistance to the pathogen. Considering the genetic origin of the resistance of the IAC genotypes, it was verified that all originated from the American cultivar Auburn 56. However, the cultivar Auburn 56 is considered obsolete in breeding programs in the USA, since it has only intermediate resistance to the Fusnem complex (Shepherd, 1982, 1983), and for many years has been replaced by other sources of resistance, such as those from Auburn 623 and 624, with high levels of resistance to *M. incognita*, although they are not commercially used for being agronomically inferior (Koenning *et al*., 2001).

Almeida *et al*. (2009), tested the cultivar IPR 140, which was released in 2007 from the line PR 94-227-9 / 18, that later became cultivar IPR 120, with excellent results in relation to multiple resistance to the main diseases of the cotton. Initial selection was made in 2000, with emphasis on productive potential and resistance/tolerance to *Rotylenchulus reniformis*. This cultivar stood out mainly against nematodes, fusariosis, angular spot, alternariosis and virus diseases. These authors studied the behavior of cultivar IPR Jataí (Almeida *et al*., 2009) obtained by genealogical selection in a hybrid population resulting from crossbreeding, between Australian and American genetic based parents. In general, it is evident that this cultivar stood out against the same diseases mentioned for cultivar IPR-140.

Another report on resistance was presented by Goldfarb *et al*. (2003), which evaluated 23 lines from the Embrapa cotton breeding program and three cultivars for the reaction to *F. oxysporum* f. sp. *vasinfectum* associated with *M. incognita* race 3. Thus, IAC-23, CNPA GO 2000-130 and CNPA GO 2000-1148 lines were resistance to the parasitism of *M. incognita*, based on number of egg masses, as well as the absence of symptoms of wilt and infection by *F. oxysporum* f. sp. *vasinfectum.*

Mechanisms of resistance in cotton

In general, two types of resistance are possible: pre-infective and post-infective. Pre-infective resistance is a passive resistance, occurring before the nematode penetration through the root surface, which is associated with the production of root exudates that are repellent or toxic to J2 or prevent penetration due to adverse physical root conditions (Rhode, 1972; Roberts *et al*., 1998). Post-infective resistance is the most common and manifests after J2 penetration into plant tissues. It is an active resistance, determined by the interaction between the parasite and the host (Roberts *et al*., 1998).

Resistance is reported as a mode of inheritance that can be expressed by a single gene (monogenic), by some genes (oligogenic), or by a larger number of genes (polygenic) (Roberts, 2002). The cellular and biochemical events associated with the resistance of cotton and other plants have been studied extensively. In most cases, J2 invades the roots of resistant plants and migrates through the tissues as in susceptible plants. However, in resistant plants, the development of the specialized giant cells required for nematode development is inhibited and the nematode is unable to complete its life cycle. In some cases, resistant plants exhibit hypersensitivity response (RH), which results in the collapse and death of cells close to the nematode. Toxic terpenoid aldehydes can accumulate around the front part of the nematode, faster in resistant than in susceptible plant (Kirkpatrick & Rothrock, 2001).

In an evaluation of the post-infection development of the nematode in the resistant genotype (M-315) Jenkins *et al*. (1995) observed that although, J2 penetration occurred normally, most of the juvenilles inside the roots of M-315 failed to develop and reach the J3 and J4 stages. After penetration into the M-315, many J2s failed to maintain the development of giant cells and only few, small galls were produced. The critical moment for nematode development in the resistant genotype occurred around six days

(transition from J3 to J4) and again at 24 days (transition from young females to adult females with eggs). At these times, the number of nematodes decreased markedly, and may be the moments when the two resistance genes are actively expressed (Creech *et al*.,1995; McPherson, 2004).

Wubben *et al*. (2008) analyzed the accumulation of transcripts of the protein MIC-3 (*Meloidogyne* Induced protein cotton 3) and the phenotypic characterization in M-315 (resistant) and M8 (susceptible) genotypes at 14, 21, 28 and 35 days after inoculation. These authors observed that at 14 days there was penetration of J2, early stages of gall formation and a significant increase in MIC-3 transcription levels in both germplasms, however the MIC-3 induction was significantly higher in the resistant genotype compared to the susceptible. At 21 days no apparent development differences were observed between infected roots of M-315 and M8, but the level of MIC-3 transcription in M315 was approximately eight times higher. At 28 days, for M-315, the size of the galls showed no increase compared to the previous time, and the galls presented dark color, in contrast to the root galls of M8, which continued to grow, showing a light color. J2 developed up to the J4 stage in M8, and there was no significant progress in the development of nematodes in M-315, and MIC-3 transcription levels decreased in M-315 roots to a level equal to M8. At 35 days, fully developed females and egg production were observed, but this development was not observed in the roots of the resistant plant (Wubben *et al*., 2008).

Histological sections performed by Mota *et al*. (2013) in resistant accession CIR 1348 (*G. barbadense* barbadense race) showed that parasitism can be discontinued after penetration or during migration of J2, between the 7th and the 21st day after inoculation. Observations in fluorescence and light field microscopy showed that cells in the proximities of the nematodes exhibited a hypersensitivity reaction with

accumulation of phenolic compounds and the presence of necrotic cells that limited the development of J2 and the formation of giant cells. Fewer giant cells were found at 21 days after inoculation with completely degenerate cytoplasmatic content, alongside of deformed nematodes.

REFERENCES

Abad P, Gouzy J, Aury J, Castagnone-Sereno P, Danchin E, *et al*., 2008. Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita. Nature Biotechnology* **26**, 909–915.

ABRAPA – Associação Brasileira dos Produtores de Algodão. 2019. Disponível em: <http://abrapa.com.br/>. Access in Jan. 2019.

Allan GJ, Max TL, 2010. Molecular genetic techniques and markers for ecological research. *Nature Education Knowledge* **3**, (10) 2

Almeida WP, Pires JR, Ruano O, Turkiewicz L, Yamaoka RS, *et al*., 2009. IPR 140: cultivar com resistência/tolerância aos nematoides *Rotylenchulus reniformis* e *Meloidogyne incognita*. VII Congresso Brasileiro do Algodão, Foz do Iguaçu, PR. 1606.

Arias MC, Infante-Malanchias ME, 2001. RFLP: o emprego de enzimas de restrição para a detecção de polimorfismo no DNA. In: Matioli SR, ed. *Biologia molecular e evolução.* São Paulo. Nobel. 143-152.

Ashkenazi V, Chani E, Lavi U, Levy D, Hillel J, Veilleux RE, 2001. Development of microsatellite markers in potato and their use in phylogenetic and fingerprinting analyses. *Genome* **44**, 50-62.

Asmus GL, 2004. Ocorrência de nematoides fitoparasitas em algodoeiro no estado de Mato Grosso do Sul. *Nematologia Brasileira* **28**, 77-86.

Beltrão NEM, de Oliveira MIP, de Sousa Júnior SP, de Brito GG, Cardoso GD, 2011. Ecofisiologia do algodoeiro (*Gossypium hirsutum* L. r. *latifolium* Hutch.). In: Beltrão NEM, de Oliveira MIP, eds., *Ecofisiologia das culturas de algodão, amendoim, gergelim, mamona, pinhão-manso e sisal*, Embrapa Informação Tecnológica, Brasília, Embrapa Algodão, Campina Grande, 65-123.

Blok VC, Phillips MS, Mcnicol JW, Fargette M, 1997. Genetic variation in tropical *Meloidogyne* spp. as shown by RAPDs. Fundamental and *Applied Nematology* **20**, 127-133.

Blok VC, Powers O, 2009. Biochemical and molecular identification. In: Perry R, Moens M; Starr JL, eds. *Root-knot Nematodes*. Cambridge, *CABI International*, 98-118.

Brondani RPV, Brondani C, Tarchini R, Grattapaglia D, 1998. Development, characterization and mapping of microsatelite markers in *Eucaliptus grandis* and *E. urophylla. Theoretical and Applied Genetics* **97**, 816-827.

Brubaker CL, Bourland FM, Wendel JF, 1999. The origin and domestication of cotton. In: Smith CW, Cothren JT, eds. *Cotton: origin, history, technology and production.* New York, John Wiley and Sons, 1-172.

Brunel, D, 1994. A microsatellite marker in *Helianthus annuus* L. *Plant Molecular Biology* **24**, 297-400.

Carneiro RMDG, Almeida MRA, Quénéhervé P, 2000. Enzyme phenotypes of *Meloidogyne* spp. populations. *Nematology* **2**, 645-654.

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

Carneiro RMDG, Neves DI, Falcão R, Paes NS, Cia E, Sá MFG, 2005, Resistência de genótipos de algodoeiro a *Meloidogyne incognita* raça 3: reprodução e histopatologia. *Nematologia Brasileira* **29**, 1-10.

Carneiro RMDG, Tigano MS, Randig O, Almeida MRA and Sarah JL, 2004. Identification and genetic diversity of *Meloidogyne* spp. (Tylenchida: *Meloidogynidae*) on coffee from Brazil, Central America and Hawaii. *Nematology* **6**, 287-298.

Carneiro RMDG, 2015. Raças de *Meloidogyne* spp.: conceitos e pesquisas futuras. Anais *XXXII Congresso Brasileiro de Nematologia*, 36.

Castagnone-Sereno P, 2002. Genetic variability of nematodes: a threat to the durability of plant resistance genes?. *Euphytica* **124**, 193-199.

Castagnone-Sereno P, Wanlerberghe-Massutti F, Leroy F, 1994. Genetic polymorphism between and within *Meloidogyne* species detected with RAPD markers. *Genome* **37**, 904-909.

Castro JMC, Lima RD, Carneiro RMDG, 2003. Variabilidade isoenzimática de populações de *Meloidogyne* spp. de regiões brasileiras produtoras de soja. *Nematologia Brasileira* **27**, 1-12.

Cenis JL, 1993. Identification of four major *Meloidogyne* spp. by random amplified polymorphic DNA (TAPD-PCR). Phytopathology **83**, 76-78.

Cofcewicz ET, Carneiro, RMDG, Randig O, Chambrier C, Quénéhervé P, 2005. Diversity of *Meloidogyne* spp. on *Musa* in Martinique, Guadeloupe and French Guiana. *Journal of Nematology* **37**, 313-322.

CONAB, 2019. Acompanhamento da safra brasileira de grãos. Quarto levantamento de safra 2018/19, Brasília, *Conab* **6**, 1-126.

Concibido VC, Young ND, Lange DA, Denny RL, Danesh D, Orf JH, 1996.Target comparative genome analysis and qualitative mapping of major partial-resistance gene to the soybean cyst nematode. *Theoretical Applied Genetics* **93**, 234-241.

Correa VR, Santos MFA, Almeida MRA, Peixoto JR, Castagnone-Sereno P, Carneiro RMDG, 2013. Species-specific DNA markers for identification of two root-knot nematodes of coffee: *Meloidogyne arabicida* and *M. izalcoensis*. *European Journal of Plant Pathology* **137**, 305-313.

Correa VR, Mattos VS, Almeida MRA, Santos MFA,Tigano MS, Castagnone-Sereno P, Carneiro RMDG, 2014. Genetic diversity of the root-knot nematode *Meloidogyne ethiopica* and development of a specie-specific SCAR marker for this diagnosis. *Plant Pathology* **63**, 476-483.

Creech RG, Jenkins JN, Tang B, Lawrence GW, McCarty JC, 1995. Cotton Resistance to Root-Knot Nematode: I. Penetration and Reproduction. *Crop Science*. **35**, 365-368.

Dalmasso A, Bergé JB, 1978. Molecular polymorphism and phylogenetic relationship in some *Meloidogyne* spp.: application to the taxonomy of *Meloidogyne. Journal of Nematology* **10**, 323-332.

De Ley P, Blaxter ML, 2002. Systematic position and phylogeny. In: Lee, DL, eds. *The Biology of Nematodes*. Taylor and Francis, London, 1-30.

Dickson DW, Huisingh D, Sasser JN, 1971. Dehydrogenases, acid and alcaline phosphatases, and esterases for chemotaxonomy of selected *Meloidogyne*, *Ditylenchus*, *Heterodera* and *Aphelenchus* spp. *Journal of Nematology* **3**, 1-16.

Eisenback JD, Triantaphyllou HH, 1991. Root-knot nematode: *Meloidogyne* spp. and races. In: Nickle, W.R ed. *Manual of Agricultural Nematology*. Marcel Dekker, Inc., New York, 191-274.

Esbenshade PR, Triantaphyllou AC, 1985. Use of enzyme phenotypes for identification of *Meloidogyne* species (Nematoda: Tylenchida). *Journal of Nematology*, **17**, 6-20.

Eisenback JD, 1983. Loss of resistance in tobacco cultivar 'NC95' by infection of *Meloidogyne arenaria* or *M. hapla*. *Journal of Nematology* **15**, 478.

Fang DD, Yu JZ, 2012. Addition of 455 microsatellite marker loci to the high density *Gossypium hirsutum* TM-1 x *G. barbadense* 3-79 genetic map. *The Journal of Cotton Science* **16**, 229–248.

Fassuliotis G, 1985. The role of the nematologists in the development of resistant cultivars. In: Sasser JN, Carter CC, eds. *An advanced treatise on Meloidogyne.* North Carolina State University Graphics, Raleigh, **1**, 233–240.

Favoreto L, Faleiro VO, Freitas MA, Brauwers LR, Galbieri R, Lopes-Caitar JAVS, Marcelino-Guimarães FC, Meyer MC, 2018. First report of *Aphelenchoides besseyi* infecting the aerial part of cotton plants in Brazil. *Disease Notes* **102**, (12) 2662.

Ferraz S, Freitas LG, Lopes EA, Dias-Arieira CR, 2010. Manejo sustentável de fitonematoides. 1ª edição. *Editora UFV*, Viçosa-MG.

Ferreira ME, Grattapaglia D, 1998. Introdução ao uso de marcadores moleculares em análise genética. 3ª edição. *Embrapa*. Brasília-DF.

Fourie H, Zijlstra C, Mcdonald AH, 2001. Identification of root-knot nematode species occurring in South Africa, using the SCAR-PCR technique. *Nematology* **3**, 675-80.

Freire EC, 2014. Melhoramento no Brasil. In: Borém A, Freire EC, eds. *Algodão: do plantio à colheita*. Viçosa, MG. 113-132.

Freitas LG, Oliveira RDL, Ferraz S, 2006. Introdução à Nematologia. 3ª edição. *Ed. UFV*. Viçosa-MG.

Goldfarb M, Amaral FV, Morello, CL, Araújo AE, Suassuna ND, 2003. Reação de genótipos de algodoeiro a *Meloidogyne Incognita* raça 3 associado a *Fusarium oxysporum* f. sp. *vasinfectum*. In: *IV Congresso Brasileiro de Algodão*, 2003, Goiânia, GO.

Galbieri R, Inomoto MM, Silva RA, 2015. Manejo de nematoides na cultura do algodoeiro em Mato Grosso. In: Belot JL, ed. *Manual de boas práticas de manejo do algodoeiro em Mato Grosso.* Cuiabá: IMAMT **2**, 214-225.

Gomez GM, Moretzohn MC, Giband M, Silva EH, Silva JGP, Furlanetto C, Carneiro RMDG, 2016. Marcadores microssatélites associados a QTLs de resistência a *Meloidogyne incognita* raça 3 em *Gossypium barbadense*. *XXXII Congresso Brasileiro de Nematologia*, 131.

Gutiérrez AO, Stelly DM, Saha S, Jenkins JN, Mccarty JC, Raska DA, Scheffler BE, 2010. SSR markers closely associated with genes for resistance to root- knot nematode on chromosomes 11 and 14 of Upland cotton. *Theoretical and Applied Genetics* **121**, (7) 1323-1337.

Hartman RM, Sasser JN, 1985. Identification of *Meloidogyne* species on the basis of differential host test and perineal pattern morphology. In: Barker KR, Carter CC, Sasser JN, eds. *An advanced treatise on Meloidogyne*. Methodology. Raleigh, NC, USA, North Carolina State University Graphics **2**, 69-77.

Hussey RS, Janssen GJW, 2002. Root-knot nematodes: *Meloidogyne* species. In: Starr, J.L.; Cook R.; Bridge, J. eds. *Plant resistance to parasitic nematodes.* CAB International, Wallingford, 43-70.

Hussey RS, Sasser JN, Huising D, 1972. Disc-electrophoretic studies of soluble proteins and enzymes of *Meloidogyne incognita* and *M. arenaria*. *Journal of Nematology* **4**, 183-189.

Hutchinson JB, 1951. Intra-specific differentiation in *Gossypium hirsutum*. *Heredity* **5**, 163-193.

Inomoto MM, 2001. Algodão: atacado por nematoides. *Revista Cultivar* **3**, (30) 5-7.

Jenkins JN, Creech RG, Tang B, Lawrence GW, McCarty JC, 1995. Cotton resistance to root-knot nematode: II. Post-penetration development. *Crop Science* **35**, 369-373.

Karssen G, 1996. Description of *Meloidogyne fallax* n. sp. (Nematoda: *Heteroderidae*), a root-knot nematode from the Netherlands. *Fundamental and Applied Nematology* **19**, 593–599.

Karssen G, Moens M, 2006. Root-knot nematodes. In: PerryRL, Moens M, eds. *Plant Nematology*. CABI Publishing, Wallingford, 59-90.

Kirkpatrick TL, Rothrock CS, 2001. Nematode fungal interactions.In: Kirkpatrick TL, Rothrock CS eds. *Compendium of Cotton Diseases.* The American Phytopathological Society. Minnesota. 68-72.

Koenning SR, Barker KR, Bowman DT, 2001. Resistance as a tatic for management of *Meloidogyne incognita* on Cotton in North Carolina. *Journal of Nematology* **33**, 2- 3:126-131.

Lewin, B, 2001. Genes VII. Artmed Editora, Porto Alegre, 955.

Litt M, Luty JA, 1989. A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *American Journal of Human Genetics* **44**, (398) 401.

Lordello AIL, Lordello RRA, 1996. Identificação de raças de *Meloidogyne incognita* associada a algumas plantas. *Summa Phytopathologica* **22**, 43-45.

Lubbers EL, Chee PW, 2009. The worldwide gene pool of *G. hirsutum* and its improvement. In: Paterson AH, ed. *Plant genetics and genomics: crops and models: Genetics and Genomics of Cotton*. Springer, New York, 23-52.

Mammadov J, Aggarwal R, Buyyarapu R, Kumpatla S, 2012. SNP Markers and Their Impact on Plant Breeding. *International Journal of Plant Genomics*,**12** 0-11.

Mattos VS, Mulet K, Cares JE, Gomes CB, Fernandez D, Grossi MF, Carneiro RMDG, Castagnone-Sereno P, 2019. Development of diagnostic SCAR markers for *Meloidogyne graminicola*, *M. oryzae*, and *M. salasi* associated with irrigated rice fields in Americas. *Plant Disease* **103**, (1) 83-88.

Mclain DKO, Rai KS, Fraser JM, 1987. Intraespecific and interspecific variation in the sequence and abundance of highly repeated DNA among mosquitoes of the *Aedes albopictus* subgroup. *Heredity* **58**, 373-381.

Maggenti A, 1981.General Nematology. Springer-Verlag, New York.

McPherson MG, Jenkins JN, Watson CE, Mccarty JC, 2004. Inheritance of root-knot nematode resistance in M-315 RNR and M78-RNR cotton. *Journal of Cotton Science* **8,** 154–161.

Meng QP, Long H, Xu JH, 2004. PCR assays for rapid and sensitive identification of three major root-knot nematodes, *Meloidogyne incognita*, *M. javanica* and *M. arenaria*. *Acta Phytopathologica Sinica* **34**, 204-210.

Moens M, Perry RN, Starr JL, 2009. *Meloidogyne* species – a diverse group of novel and important plant parasites. In: Perry RN, Moens M, Starr JL eds. Root-knot nematodes. CAB International, Wallingford, 1-17.

Mohan M, Nair S, Bhagwat A, Krishna TG, Yano M, Bhatia CR, Sasaki T, 1997. Genome mapping, molecular markers and marker-assisted selection in crop plants *Molecular Breeding* **3**, 87–103.

Mota FC, Alves GCS, Giband M, Gomes ACMM, Sousa FR, Mattos VS, Barbosa VHS, Barroso PAV, Michel N, Peixoto JR, Rocha MR, Carneiro RMDG,2013. New sources of resistance to *Meloidogyne incognita* race 3 in wild cotton accessions and histological characterization of the defense mechanisms. *Plant Pathology* **62**, (5) 1173-1183.

Moura RM, 1996. Gênero *Meloidogyne* e a meloidoginose. Parte I. *Revisão Anual de Patologia de Plantas* **4**, 209-245.

Niu C, Hinchliffe DJ, Cantrell RG, Wang C, Roberts PA, Zhang J., 2007. Identification of molecular markers associated with root-knot nematode resistance in upland cotton. *Crop Science* **47**, (3) 951-960.

Noir S, Anthony F, Bertrand B, Combes MC, 2003. Identification of a major gene (Mex-1) from *Coffea canephora* confering resistance to *Meloidogyne exigua* in *Coffea arabica*. *Plant Pathology* **52**, 97-103.

Ogallo JL, Goodell PB, Eckert J, Roberts PA, 1997. Evaluation of NemX, a new cultivar of cotton with high resistance to *Meloidogyne incognita*. *Journal of Nematology* **29**, (4) 531-537.

Paran I, Michelmore RW, 1993. Development of reliable PCR–based markers linked to downy mildew resistance genes in lettuce. *Theoretical and Applied Genetics* **85**, 985-993.

Percy GR, 2009. The worldwide gene pool *of G. barbadense* and its improvement. In: Paterson AH (Ed.) Plant genetics and genomics: crops and models: genetics and genomics of cotton. New York, Springer. 53-68.

Percy RG, Wendel JF, 1990. Allozyme evidence for the origin and diversification of *Gossypium barbadense* L. *Theoretical and Applied Genetics* **79**, (4) 529-542.

Perina FJ, Maximiniano C, Rhode A, dos Santos IA, Freire E, Lopes CML, 2017. Primeira ocorrência de ataque de nematoide de parte aérea, *Aphelenchoides* sp., em algodoeiro no Estado da Bahia. In: *11 Congresso Brasileiro do Algodão*, Maceió. Resumos.

Ploeg A, Maris P, 1999. Effects of temperature on the duration of the life cycle of a *Meloidogyne incognita* population. *Nematology* **1**, 389-393.

Randig O, Bongiovanni M, Carneiro, RM, Castagnone-Sereno P, 2002. Genetic diversity of root-knot nematodes from Brazil and development of SCAR markers specific for the coffee-damaging species. *Genome* **45**, (5) 862-870.

Rhode RA, 1972. The expression of resistance in plants to nematode. *Annual Review of Phytopathology* **10**, 233-252.

Riggs RD, Winstead NN, 1959. Studies on resistance in tomato to root-knot nematodes and on the occurrence of pathogenic biotypes. *Phytopathology* **49**, 716- 724.

Roberts PA, 2002. Concepts and consequences of resistance. In: Starr JL, Cook R, Bridge J, eds. *Plant resistance to parasitic nematodes*. CAB International. Wallingford. 25-41.

Roberts PA, Mattews WC, Veremis JC, 1998. Genetic mechanisms of host-plant resistance to nematodes. In: Pederson GA, Windham GL, Barker KR, eds. *Plant Nematode interactions*. Agronomy Society of America, Wisconsin. 209- 238.

Robinson AF, Bowman DT, Cook CG, Jenkins JN, Jones JE, May LO, et al. 2001. Nematode resistance. In KirkpatrickTL, Rothrock CS eds. *Compendium of cotton diseases* Saint Paul, MN: APS Press, 68-72.

Rodrigues CS, Bueno MG, 2004. *A saga do algodão: das primeiras safras à ação na OMC*. Insight Engenharia, Rio de Janeiro, 144.

Santos MFA, Furlanetto C, Almeida MRA, Carneiro MDG, Mota FC, Mendes ACM, Silveira NOR, Silva JGP, Castagnone-Sereno P, Tigano MS, Carneiro RMDG, 2012. Biometrical, biological, biochemical and molecular characteristics of *Meloidogyne incognita* isolates and related species. *European Journal of Plant Pathology* **134**, 671-684.

Schuster I, Abdelnoor RV, Marin SRR, Carvalho VP, Kiihl RAS, Silva JFV, Sediyama CS,Barros EG,Moreira MA, 2001. Identification of a new major QTL associated with resistance to soybean cyst nematode (*Heteroderaglycines*). *Theoretical and Applied Genetics* **102**, 91-96.

Semblat JP, Wajnberg E, Dalmasso A, Abad P, Castagnone-Sereno P, 1998. High-resolution DNA fingerprinting of parthenogenetic root-knot nematodes using AFLP analysis. *MolecularEcology* **7**, 119–125.

Shen X, He Y, Lubbers EL, Davis RF, Nichols RL, Chee PW, 2010. Fine mapping QMi-C11 a major QTL controlling root-knot nematodes resistance in Upland cotton. *Theoretical and Applied Genetics* **121**, (8) 1623-1631.

Shen X, Van Becelaere G, Kumar P, Davis RF, May OL, Chee P, 2006. QTL mapping for resistance to root-knot nematodes in the M-120 RNR Upland cotton line (*Gossypium hirsutum* L.) of the Auburn 623 RNR source. *Theoretical and Applied Genetics* **113**, (8) 1539-1549.

Shepherd RL, 1974a. Registration of Auburn 623 RNR cotton germplasm. *Crop Science* **14**, 911.

Shepherd RL, 1974b. Transgressive segregation for root-knot nematode resistance in cotton. *Crop Science* **14** ,872-875.

Shepherd RL, 1982. Registration of three germplasm lines of cotton (Reg. Nos. GP 164–166). *Crop Science* **22**, 692.

Shepherd RL, 1983. New sources of resistance to root-knot nematodes among primitive cottons. *Crop Science* **23**, 999–1002.

Silva CM, Santos MA, 1997. Levantamento de nematoides na cultura do algodoeiro. *Nematologia Brasileira* **21**, (1) 22-23.

Silva EH, Mattos VS, Furlanetto C, Giband M, Barroso PAV, Moita AW, Jorge-Junior A, Correa VR, Castagnone-Sereno P, Carneiro RMDG, 2014. Genetic variability and virulence of *Meloidogyne incognita* populations from Brazil to resistant cotton genotypes. *European Journal of Plant Pathology* **139**, (1) 195-204.

Silva RA, Serrano MAS, Inomoto MM, Asmus GL, 2004. Distribuição, dinâmica populacional e danos provocados por *Meloidogyne incognita*, *Rotylenchulus reniformis* e *Pratylenchus brachyurus* na cultura do algodoeiro no estado do Mato Grosso. *Grupo de Produção Acadêmica de Ciências Agrárias e Biológicas.* UNIVAG, Várzea Grande, 36.

Starr JL, Page LJ, 1993. Nematode parasites of cotton and other tropical fibber crops. In: Luc M, Sikora RA, Bridge J, eds. *Plant Parasitic nematodes in subtropical and tropical agriculture.* CAB International, Wallingford, 539-556.

Stuber CW, Goodman MM, Moll RH, 1982. Improvement of yield and ear number resulting from selection at allozyme loci in a maize population. *Crop Science* **22**, (4) 737-740.

Suassuna ND, Scoz LB, Giband M, 2016. Melhoramento genético do algodoeiro para resistência aos nematoides: seleção assistida por marcadores moleculares. In: Ghalbieri R, Bellot JL, eds. *Nematoides fitoparasitas do algodoeiro nos cerrados brasileiros: biologia e medidas de controle.* Cuiabá: Instituto Mato-grossense do Algodão, 243-256.

Tanksley SD, 1993. Mapping polygenes. *Annual Reviews of Genetics* **27**, 205-233.

Taylor AL, Sasser JN, 1978. Biology, identification and control of root-knot nematodes (*Meloidogyne* species). Raleigh. North Carolina State University Graphics.

Taylor DT, Sasser JN, 1983. Biologia, identificación y control de los nematodos de nódulo de la raiz (*Meloidogyne* species). North Carolina State University and USAID.

Tigano MS, Carneiro RMDG, Jeyaprakash A, Dickson DW, Adams B, 2005. Phylogeny of *Meloidogyne* spp. based on 18S rDNA and the intergenetic region of mitochondrial DNA sequences. *Nematology* **7**, 851–862.

Tigano M, Siqueira K, Castagnone-Sereno P, Mulet K, Queiroz P, Santos M, Teixeira C, Almeida M, Silva J, Carneiro R, 2010. Genetic diversity of the root-knot nematode *Meloidogyne enterolobii* and development of SCAR marker for this guava-damaging species. *Plant Pathology* **59**, 1054-1061.

Trudgill DL, Blok VC, 2001. Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. *Annual Review of Phytopathology* **39**, 53-77.

U.S. Department of Agriculture, 2018. The world and United States cotton outlook. In: Johnson J, MacDonald S, Meyer L, Ston L, eds.www.usda.gov/oce/forum Access in Jan. 2019.

Veech JA, Starr JL, 1986. Comparison of development, reproduction, and aggressiveness of *Meloidogyne incognita* races 3 and 4 on cotton. *Journal of Nematology* **18**, **(**3) 413-415.

Westengen OT, Huamán A, Heun M, 2005. Genetic diversity and geographic pattern in early South America cotton domestication. *Theoretical and Applied Genetics* **110**, **(**2) 392-402.

Wubben MJ, Callahan FE, Hayes RW, Jenkins JN, 2008. Molecular characterization and temporal expression analyses indicate that the MIC (*Meloidogyne* induced cotton) gene family represents a novel group of root-specific defense-related genes in upland cotton (*Gossypium hirsutum* L.). *Planta* **228**, 111-123.

XU, Y.; Crouch, JH. 2008. Marker-assisted selection in plant breeding: from publications to practice. *Crop Science* **48**, 391-407.

Ynturi P, Jenkins JN, Mccarty JC, Gutierrez OA, Saha S, 2006. Association of root-knot nematode resistance genes with simple sequence repeat markers on two chromosomes in cotton. *Crop Science* **46**, (6) 2670-2674.

Yu Y, Yuan D, Liang S, Li X, Wang X, 2011. Genome structure of cotton revealed by a genome-wide SSR genetic map constructed from a BC1 pulation between *Gossypium hirsutum* and *G. barbadense*. *BMC Genomics* **12**, 15.

Zhang Y, Lin ZX, Xia Q, Zhang M, Zhang XL, 2008. Characteristics and analysis of simple sequence repeats in the cotton genome based on a linkage map constructed from a BC1 population between *Gossypium hirsutum* and *G. barbadense*. *Genome* **51**, 534–546.

Zhou E, Starr JL, Smith CW, 1999. Inheritance of resistance to *Meloidogyne incognita* in the cotton cultivar AcalaNemX. *Journal of Nematology* **31**, 584-585.

Zhou E, Wheeller TA, Starr JL, 2000. Root galling and reproduction of *Meloidogyne incognita* isolates from Texas on resistant cotton genotypes. *Journal of Nematology* **32**, 513-518.

Ziljstra C, Donkers-Venne DTHM, Fargette M, 2000. Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterised amplified regions (SCAR) based PCR assays. *Nematology* **2**, 847-853.

CHAPTER 2

Diversity of *Meloidogyne incognita* populations from cotton and aggressiveness to *Gossypium* spp. accessions

ABSTRACT

The root-knot nematode (RKN) *Meloidogyne incognita* is the main nematode causing losses to the cotton crop in Brazil. In order to implement control strategies within integrated management, an accurate identification of the nematode populations prevailing in the cotton growing areas is necessary. This study aimed to assess the genetic variability and aggressiveness of RKN populations from cotton production areas in Bahia State, Brazil. All populations were characterized biochemically and molecularly, and identified as *M. incognita*. RAPD and AFLP markers detected 44% of polymorphic fragments among 13 populations of this species. The 10 *M. incognita* populations collected in Bahia presented a diversity of 33.7% when compared to each other and 25% when the population from Barreiras (the most polymorphic) was excluded. This polymorphism increased when populations from other Brazilian states were included. The aggressiveness/virulence among populations from Bahia towards different cotton accessions (susceptible/resistant) was also studied. None of the populations showed virulence against the moderately resistant (Clevewilt-6, Wild Mexican Jack Jones and LA-887) and the highly resistant (CIR1348, and M-315 RNR) cultivars. Two populations of *M. incognita* from Barreiras were the most aggressive reaching reproduction factors (RF) of 539 and 218, respectively in the susceptible cultivar FM 966. The most aggressive population (8) was also the most genetically

divergent in phylogenetic analyses. These results demonstrate that diversity of *M. incognita* populations from cotton farms in Bahia was not related to virulence against resistant accessions, which suggest that cultivars containing a single or two resistant genes with good agronomic characteristics can be used in infested commercial areas in Bahia State, Brazil.

Keywords: root-knot nematode, AFLP, RAPD, RKN management, resistance, pathogenicity.

RESUMO

Diversidade de populações de *Meloidogyne incognita* do algodoeiro e agressividade em acessos de *Gossypium* spp.

O nematoide das galhas (NDG) *Meloidogyne incognita* é o principal nematoide causador de perdas para a cultura do algodão no Brasil. A fim de implementar estratégias de controle no manejo integrado, é necessária a identificação precisa das populações de nematoides prevalentes nas áreas de cultivo de algodão. O objetivo deste estudo foi avaliar a variabilidade genética e a agressividade de populações do NDG de áreas de produção de algodão no Estado da Bahia, Brasil. Todas as populações foram caracterizadas bioquimicamente e molecularmente e identificadas como *M. incognita*. Os marcadores RAPD e AFLP detectaram 44% de fragmentos polimórficos entre 13 populações desta espécie. As 10 populações de *M. incognita* coletadas na Bahia apresentaram uma diversidade de 33,7% quando comparadas entre si e 25% quando a população de Barreiras (a mais polimórfica) foi excluída. Esse polimorfismo aumentou quando populações de outros estados brasileiros foram incluídas. A agressividade/virulência entre populações da Bahia para diferentes acessos de algodão

(suscetível/resistente) também foi estudada. Nenhuma das populações apresentou virulência contra as cultivares moderadamente resistentes (Clevewilt-6, Wild Mexican Jack Jones e LA-887) e as altamente resistentes (CIR1348 e M-315 RNR). Duas populações de *M. incognita* de Barreiras foram as mais agressivos, com fatores de reprodução (FR) de 539 e 218 respectivamente, na cultivar suscetível FM 966. A população mais agressiva (8) também foi a mais geneticamente divergente nas análises filogenéticas. Estes resultados demonstram que a diversidade de populações de *M. incognita* provenientes de lavouras de algodão na Bahia não está relacionada à virulência contra acessos resistentes, sugerindo que cultivares contendo um ou dois genes de resistência com boas características agronômicas podem ser utilizadas em áreas comerciais infestadas no Estado da Bahia, Brasil.

Palavras-chave: Nematoide das galhas, AFLP, RAPD, manejo de nematoide das galhas, resistência, patogenicidade.

1. INTRODUCTION

Several diseases and pests affect cotton (*Gossypium* spp.) yield worldwide. In Brazil, the nematode causing the greatest yield losses in the cotton crop is the root-knot nematode (RKN) *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949, due to its wide distribution, survival capacity, and a wide range of host plants. The yield losses caused by this RKN species are higher in sandy soils with low fertility, and when in association with the cotton wilt-causing agent, the fungus *Fusarium oxysporum* f. sp. *vasinfectum*, inducing the Fusarium–nematode disease complex (Wang & Roberts, 2006). The occurrence of high levels of *M. incognita* populations can make cotton cultivation unfeasible, with reports of abandonment of infested areas in São Paulo,

Paraná and Goiás states (Galbieri *et al*., 2015). Bahia is the second largest cotton-growing state in Brazil, with 96% of the production in the western region, and has suffered severe losses due to the attack of this nematode (CONAB, 2018; Perina *et al*. 2017). In Brazil, most commercial cotton cultivars are susceptible to this RKN species, and only a few are moderately resistant or resistant (Silva *et al*., 2014).

The cotton American line Auburn 623 RNR resistant to *M. incognita* has been available for over 40 years (Shepherd, 1974), from which the M-315 RNR line is derived, widely used in studies as a source of high level resistance to this pathogen. The resistance observed in Auburn 623 RNR has an oligogenic inheritance that is determined by at least two genes located on chromosomes 11 and 14. This line originated from the cross between two moderately resistant accessions: Clevewilt-6 and Wild Mexican Jack Jones (WMJJ) (PI593649). The quantitative trait locus qMi-C11, originating from Clevewilt-6, has an additive gene effect and is located in the CIR069-CIR316 interval on chromosome 11, whereas QTL qMi-C14, originated from WMJJ, has an additive-dominant gene effect and is located in the BNL3545–BNL3661 interval on chromosome 14 (Shepherd, 1974; McPherson *et al*., 2004; Jenkins *et al*., 2012; He *et al*., 2014). New sources of resistance are desirable for the development of cultivars with higher levels of resistance to this RKN and less likely to be supplanted by variants of the pathogen. Recently, the accession of *G. barbadense* (CIR1348) was identified as a new source of high level resistance to *M. incognita* (Mota *et al*., 2013; Silva *et al*., 2014). In accession CIR1348 two QTLs were identified, one on chromosome 11 and another on chromosome 15, which are responsible for the high level of resistance to the nematode (Silva *et al*., 2014; M. Giband, personal communication).

The use of resistant cultivars as a strategy in integrated management requires the correct characterization and identification of the nematode populations prevalent in the

areas of cotton production, and the characterization of intraspecific genetic diversity among *M. incognita* populations is important. Within *M. incognita* species there are physiological races; however, only races 3 and 4 parasitize cotton, with race 3 found most often in commercial production areas (Inomoto, 2001). Although, the occurrence of races is recognized in *M. incognita*, Moens *et al*. (2009) recommended discontinuation of this terminology, because a small variation among populations of the same species is measured and the range of hosts is very large. The analysis of the aggressiveness and virulence of the nematode populations to a given crop would be the most appropriate strategies (Carneiro, 2015).

Parthenogenetic *Meloidogyne* species such as *M. arenaria* (Neal, 1889) Chitwood, 1949, *M. incognita* and *M. javanica* (Treub, 1885) Chitwood, 1949 have a genetic variability that allows rapid adaptation to unfavorable conditions, such as adaptation to resistant host plants (Trudgill & Blok, 2001). Reports on resistance breakdown in natural nematode populations demonstrate the ability of the pathogen to develop mechanisms of adaptation to resistance genes in the case of continuous use of the same source of resistance (Castagnone-Sereno, 2002). The selection of *M. incognita* virulent populations after successive resistant cotton plantations has occurred in California (Ogallo *et al*., 1997) and Texas (Zhou *et al*., 2000).

With the aim of contributing to breeding programs for resistance of cotton to *M. incognita*, the objective of this study was to characterize the intraspecific genetic diversity and aggressiveness/virulence of *M. incognita* populations prevailing in cotton growing areas in the western region of Bahia State, Brazil. The development and use of cotton cultivars resistant to this RKN species could reduce quantitative and qualitative losses of fiber, in addition to representing an important management strategy in cotton infested areas.

2. MATERIALS AND METHODS

2.1 Characterization and identification of RKN species and races

Ten populations of *M. incognita* were collected in infested cotton (*Gossypium* spp.) farms in western Bahia (Table 2) and multiplied in tomato (*Solanum lycopersicum* L. 'Santa Clara') plants.

Table 2. List of *Meloidogyne incognita* populations and *M. enterolobii* (M. ent, outgroup) their origin (Brazilian municipalities and States), races and esterase phenotypes (Est).

Population code	Origin	Races	Est
1	Luiz Eduardo Magalhães – BA	3	I2
2	Luiz Eduardo Magalhães – BA	3	I2
3	Luiz Eduardo Magalhães – BA	3	I2
4	São Desiderio – BA	3	I2
5	Barreiras – BA	3	I2
6	São Desiderio – BA	3	I2
7	São Desiderio – BA	4	I2
8	Barreiras – BA	3	I2
9	Luiz Eduardo Magalhães – BA	3	I2
10	Barreiras – BA	3	I2
11	Londrina – PR	3	I1
12	Umuarama – PR	3	I2
13	Dourados – MS	3	I2
M. ent	Petrolina – PE	-	VS1-S1

After four months, females were removed from the tomato roots, then identified using esterase (EST) profiles, according to the protocol described by Carneiro &

Almeida (2001), in which approximately 25 females of each *M. incognita* population were individually extracted from the tomato roots with the aid of a stylet under a stereoscopic microscope and transferred for microhematocrit tubes containing 3 µl of the extraction buffer (sucrose / Triton X-100). The tubes were kept in a container with ice throughout the sample extraction process. The females were macerated with a rounded-end steel rod and then applied, with the aid of a Hamilton syringe on Whatman 3 mm paper, 1.5 x 4.0 mm in size and then placed in the wells of the polyacrylamide gel . A pure population of *M. javanica* was used as standard in esterase phenotypes (EST). Droplets of 0.1% bromophenol blue were placed on the samples to monitor the migration. The migration in the gel underwent 80-120 V under refrigeration for approximately 2 hours.

Band patterns on the polyacrylamide gel were revealed with the isoenzyme esterase specific revealing solution (alpha-naphthyl acetate, fast blue RR salt and sodium phosphate buffer) prepared just prior to use. After incubation in the dark at 37 °C for 40 minutes the gels were washed in running water and fixed in a solution composed of distilled water, methyl alcohol and acetic acid in the ratio (5: 5: 1) (v / v) for 30 minutes. Then the gels were dried between sheets of cellophane paper and the generated profiles were analyzed and compared with those of Figure 2.

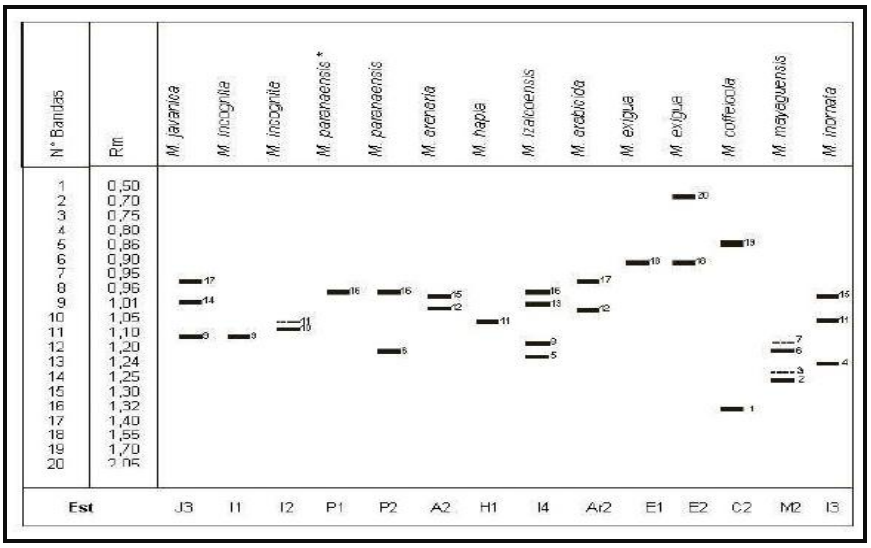

Figure 2. Isoenzyme esterase patterns for *Meloidogyne* species (Carneiro & Cofcewicz, 2008).

The races of *M. incognita* were determined according to Hartman & Sasser (1985). In which four seedlings of tomato (*Solanum lycopersicum* cv. Rutgers), tobacco (*Nicotiana tabacum* cv. NC 95), cotton (*Gossypium hirsutum* cv. Deltapine 61), pepper (*Capsicum annuum* cv. Early California Wonder), watermelon (*Citrullus vulgaris* cv. Charleston Gray) and peanut (*Arachis hypogaea* cv. Florunner), were inoculated with 10,000 eggs of each *M. incognita* population. The evaluation of plants was performed 90 days after inoculation, the Reproduction Factor (RF), Gall Index (GI) and Egg Masses Index (EMI) were evaluated, and the races were classified according to the scale of Taylor & Sasser (1978) (Table 1).

2.2 Extraction of eggs and genomic DNA for genetic diversity and SCAR markers studies

Extraction of eggs

The extraction of eggs from each population was done according to Hussey & Barker (1973) in which, the tomato roots were washed, cut and triturated in a blender

with 500 ml of 1.25% sodium hypochlorite solution (NaOCl) for 40 seconds. The triturated was then passed through a set of 60, 100 and 500 mesh overlapping sieves. The material trapped in the 500 mesh sieve was washed in tap water, collected, and dispensed into 50 ml falcon tubes, to which approximately 5 grams of kaolin were added, then centrifuged at 2500 rpm for 5 minutes. After centrifugation, the supernatant was discarded, and a cold 30% sucrose solution was added to the tubes, after homogenization, new centrifugation was done at 2500 rpm for 2 minutes. The supernatant was then passed through a 500 mesh sieve and washed with distilled water to remove the sucrose residue.

The eggs trapped in the 500 mesh sieve were transferred to 15 ml falcon tubes, which were centrifuged at 2500 rpm for 3 minutes. The water was discarded with a pipette. Then the pellet formed in each falcon tube (15 ml) was dispensed into 1.5 ml eppendorf tubes, and centrifuged at 13000 rpm for 2 minutes to separate the remaining water from the eggs. The tubes were identified and stored at -80 ° C for further extraction of the genomic DNA.

Extraction of genomic DNA

Total genomic DNA was extracted according to the method described by Randig *et al.,* (2002). The eggs previously extracted and stored at -80 ° C were macerated in a porcelain mortar with liquid nitrogen and recovered in a 1.5 ml Eppendorf tube, to which 500 µl of NIB (0.1 M NaCl, 30 mM Tris pH 8; 10 mM EDTA; 0.7 mM β-mercaptoethanol; 5 mM Triton-NPHO). After homogenization, the samples were centrifuged at 14000 rpm for two minutes, and the supernatant discarded. This step was performed twice. It was added 800 µl of homogenization buffer (0.1 M NaCl, 0.2 M sucrose, 10 mM EDTA) and 200 µl of the lysis buffer (0.125 M EDTA, 0.5 M Tris pH

9.2, 2,3% SDS), the tubes were homogenized and incubated in a water bath at 55 °C for 30 minutes, followed by 10 minutes at room temperature.

Purification of the DNA was performed by adding 1 V of phenol (1 ml) to the sample which was homogenized and centrifuged at 14000 rpm for three minutes. The supernatant was recovered and then admixed to ½ V phenol (0.5 ml) + ½ V Chloroform (0.5 ml) and centrifuged at 14000 rpm for three minutes. The supernatant was recovered in a new tube, adding 200 µl of ether, and centrifuging at 14000 rpm for three minutes. The ether was removed with the aid of a pipette.

For precipitation of the DNA, 1 ml of absolute ethanol was added to the tube, followed by homogenization. The tube was cooled to -80 °C for 30 minutes. Then, centrifugation was done at 14000 rpm for 10 minutes. The supernatant was discarded, and 70% ethanol was added. After centrifugation at 14,000 rpm for five minutes, 70% ethanol was discarded. The precipitate was dried at room temperature, recovered by volume of 20 µl of sterile water (Milli-Q). DNA was quantified and then stored at (-20 °C).

2.3 Identification of *Meloidogyne* species by SCAR markers.

Confirmation on the identification and purity of the inoculum was done using the SCAR species specific markers *Meloidogyne incognita* Inck14 (Randig *et al*., 2002) and *M. javanica* Jav (Ziljstra *et al*., 2000). The choice of the *M. javanica* marker was due to the high incidence of this species in the region, especially in areas where soybean is grown in succession to cotton.

PCR reactions were performed in a final volume of 25 µl containing 2 µl genomic DNA [3 ng / µl], 1 µl primer F (Operon Technologies, Alameda, CA, USA), 1 µl primer R (Operon Technologies (Invitrogen®, São Paulo, Brazil), 2.5 µl of 1X + MgCl 2 reaction buffer (Invitrogen), 4 µl of 1.25 mM dNTPs (dATP, dTTP, dGTP and

dCTP) São Paulo, Brazil), 0.25 µl of Taq DNA polymerase (Invitrogen®, São Paulo, Brazil) and 14.25 µl of Milli-Q water.

For the amplifications, the following programs were used according to the conditions described for each set of primers: *M. incognita*: Inck14 F / R 399 bp (Randig *et al*., 2002): initial DNA denaturation for 5 minutes at 94 °C, 35 cycles of 30 seconds at 94 °C, 45 seconds at 64 °C, 1 minute at 70 °C and final extension of 8 minutes at 70 °C. For *M. javanica*: Jav F / R 690 bp (Ziljstra *et al*., 2000): initial DNA denaturation for 2 minutes at 94 °C, 35 cycles of 30 seconds at 94 °C, 30 seconds at 64 °C, 1minute at 72 °C and final extension of 8 minutes at 72 °C. The amplified products were separated by electrophoresis in 1.5% agarose gel at constant current of 100 mA for approximately 3 hours and stained with ethidium bromide (0.3 µg / ml) and visualized under UV-visible transilluminator.

2.4 Characterization of genetic diversity of *M. incognita* by RAPD markers.

In order to compare with a previous study of genetic diversity of Brazilian *M. incognita* populations, three populations from other states: Paraná (PR) and Mato Grosso do Sul (MS), studied by Silva *et al*. (2014), (populations 11, 12 and 13) were added, and a population of *M. enterolobii* was used as outgroup (Table2). RAPD reactions occurred in a volume of 13µl containing 9 ng genomic DNA under the conditions described by Carneiro *et al*. (2008) as follow: 5 minutes at 94 °C, 40 cycles at 94 °C for 30 seconds, 36 °C for 45 seconds, 70 °C for 2 minutes and a final extension of 10 minutes at 70 °C. Forty random 10-mer oligonucleotide primers (Operon Technologies)(A12, AB1, AB06, AD03, AG04, AU13, C7, C9, F06, G2, G4, G13, J20, K10, K19, L19, M20, N7, N10, P02, P1, P6, Q10, R3, R4, R7, S20, T6, U05, V2, V7, V17, W05, W6, W15, X20, Y05, Y16, Z4, Z17) were used in the analysis. Fragments

amplified by PCR-RAPD were separated by 1.5% agarose gel electrophoresis at constant current of 100 mA for approximately 3 hours, stained with ethidium bromide (0.3 μg / ml) and visualized under transilluminator UV-visible, with samples in duplicates.

2.5 Characterization of genetic diversity of *M. incognita* by AFLP markers.

Approximately 1 μl of genomic DNA was digested by the restriction enzyme EcoRI, adaptors were attached to the ends of the fragments in a final volume of 20 μl and incubated overnight at 37 °C following recommendations of Suazo & Hall (1999). The digestion-ligation reactions were diluted with Tris-EDTA buffer to a final volume of 200 μl and stored at -20 °C. A series of thirteen 19-mer primers (Integrated DNA Technologies) were used, consisting of EcoRI adapter core sequence 5'-GACTGCGTACCAATTCAGT-3' plus the selective nucleotides AGT, ACT, ATT, GCG, CAG, TGG, CCT, ACC, GCC, CGA, CAT, CTC and CCG. The amplified fragments were separated by electrophoresis on high resolution 1.5% gel agarose-synergel (Diversified Biotech Synergel ™) as described by Semblat *et al*. (1998).

2.6 Phylogenetic analysis.

For each marker type, the amplified fragments were recorded as present or absent from the digitized photographs of the gels, and those data were converted into a binary matrix. Phylogenetic reconstruction was performed using the unweighted pair group method with arithmetic mean (UPGMA), implemented in PAUP version 4b10 (Swofford, 2002). The stability of the dendrogram nodes was tested by 1000 bootstrap replicates. The percentage of polymorphisms was calculated based on the presence of

polymorphic and monomorphic bands in the binary matrices using the formula: P = P / (P + M) * 100, where P = polymorphic bands and M = monomorphic bands.

2.7. Aggressiveness/virulence of *M. incognita* populations on cotton accessions

Gossypium accessions

The accessions of *G. hirsutum* and *G. barbadense* used in this study were obtained from Embrapa Cotton's Germplasm Collection (Table 3). These accessions were previously studied showing moderate to high resistance to populations of *M. incognita* races 3 and 4 (Mota *et al*., 2013; Silva *et al*., 2014). *Gossypium hirsutum* cv. FiberMax966 (FM966) was used as a susceptible control, while *G. hirsutum* breeding line M-315 RNR was used as a resistant control.

Table 3. Accessions of *Gossypium* spp. used in the study.

Accession name	Species	Origin – accession number
CIR1348	*G. barbadense* race barbadense	Peru – wild accession; Cirad n° CIR1348
Clevewilt-6	*G. hirsutum*	USA – obsolete cultivar with the resistance locus qMi-C11 to RKN.
Wild Mexican Jack Jones (WMJJ)	*G. hirsutum*	México – wild accession, with the resistance locus qMi-C14 to RKN. NPGS PI n° 593649
LA-887	*G. hirsutum*	USA – obsolete cultivar with resistance to RKN
M-315 RNR	*G. hirsutum*	USA – breeding line with the resistance loci qMi-C11 and qMi-C14 to RKN
Fibermax 966 (FM966)	*G. hirsutum*	Australia – commercial variety susceptible to RKN

Inoculum of Meloidogyne incognita

Six of the 10 populations collected in the state of Bahia were selected for the study of aggressiveness/virulence, based on the information of genetic variability and geographic distribution. Prior to inoculation, the populations were multiplied on tomato cv. Santa Clara for 3 months under greenhouse conditions. Eggs were extracted from infected roots using 0.5 % NaOCl, according to Hussey & Barker (1973), using a blender instead of manual agitation. Counting was done using a light microscope and Peter's slides.

Evaluation of nematode resistance in green house conditions

Seven plants of each accession 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 5,000 eggs of *M. incognita* by pipetting nematode suspension around the stem base. Plants were arranged in a randomized block design with seven replications and were watered and fertilized as needed. Three months after inoculation, the root systems were rinsed under tap water and weighed. Roots were stained with Floxin B and evaluated for gall and egg mass indexes, 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 & Sasser, 1985). Eggs were extracted by Hussey & Barker (1973) methodology, using a blender instead of manual agitation and 1 % NaOCl. The reproduction factor (RF) was calculated as RF = FP/IP, where FP = final nematode population and IP = initial nematode population (IP = 5,000). The average RF was transformed as log (x+1), submitted to analysis of variance and the means grouped using Scott-Knot's test ($P<0.05$).

3. RESULTS

3.1 Characterization of nematode populations.

All populations collected in cotton plantations in western Bahia presented the esterase profile of *M. incognita* EST I2 with two bands, a major band (Rm: 1.1) and a minor (Rm 1.2) (Figure 3a, Table 2). The specific SCAR markers of *M. incognita* (Inck14) and *M. javanica* (Jav) confirmed the identification and purity of *M. incognita* populations (Figure 3b). The 10 *M. incognita* populations from Bahia varied in their response to resistant tobacco 'NC95', population 7 from São Desiderio reproduced on tobacco and was classified as belonging to race 4, while populations 1,2,3,4,5,6,8,9,10 did not, and were assigned to race 3 (Table2).

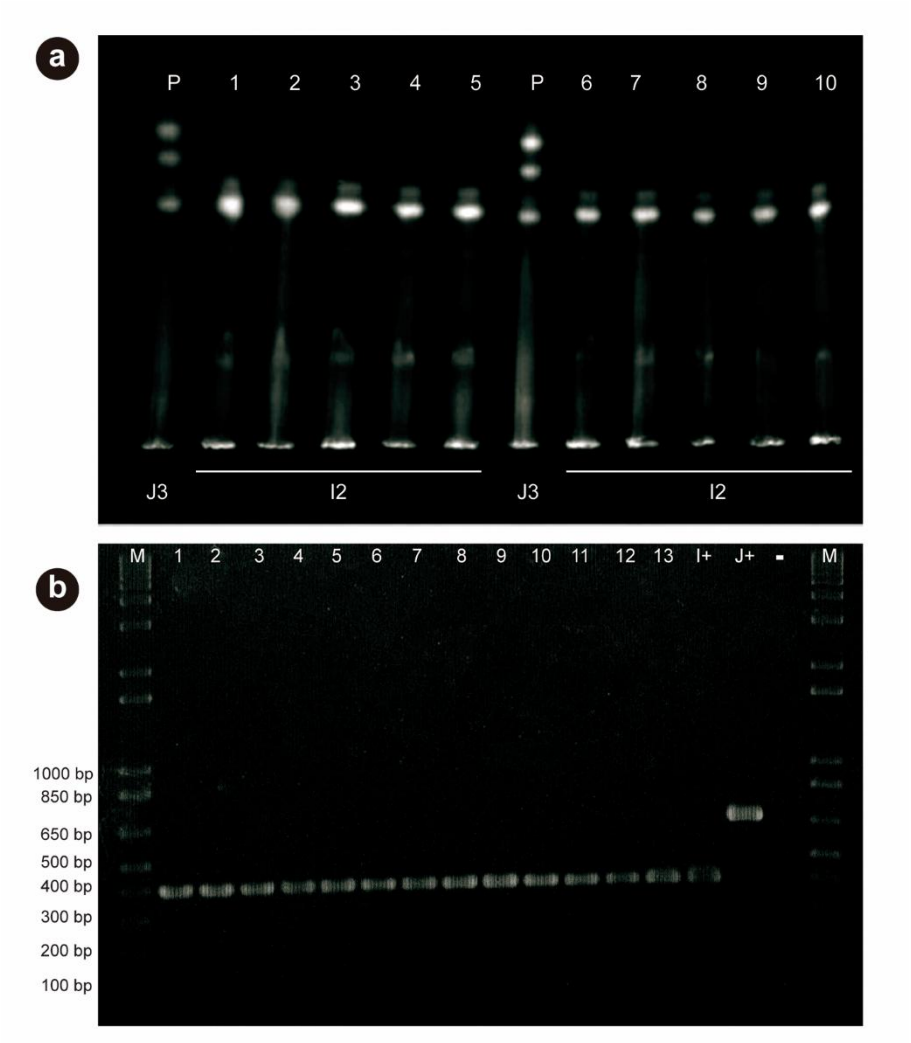

Figure 3. a- Esterase phenotypes of *Meloidogyne incognita* (EST I2) 1 – 10: isolates from Bahia. P: *M. javanica* pattern (EST J3) included as reference. **b**- PCR amplification patterns of *Meloidogyne* spp. generated with specific SCAR primers inc-K14-F/R (Randig *et al*., 2002). 1 – 10: isolates from Bahia; 11 and 12: isolates from Paraná; 13: isolate from Mato Grosso do Sul; (I+, J+): positive controls, *M. incognita* and *M. javanica*, respectively. (-) DNA: negative control. M: 1 kb Plus DNA ladder (Invitrogen), bp: base pairs.

3.2 Genetic diversity of *Meloidogyne incognita*.

The number of reproducible amplified fragments varied from 10 to 30 per primer and their size ranged from 200 to 4000 bp, a total of 820 amplified fragments were scored for both RAPD and AFLP markers (Figure 4a and 4b), of them 361 were polymorphic. All scorable amplified bands were recorded to build a 0–1 matrix, on which cluster analysis were done using UPGMA. Our results showed 44% of polymorphic fragments among all 13 populations, using RAPD and AFLP markers. The ten populations of *M. incognita* collected in Bahia presented a diversity of 33.7% when compared to each other and 25% when excluded the population 8 from Barreiras, which was the most polymorphic. This polymorphism increased when populations from other Brazilian states PR and MS were included (Table 4). The dendrogram resulting from the concatenation of RAPD and AFLP dataset is shown in Figure 5. All populations of *M. incognita* clustered together with 89% bootstrap support, however the populations 8 from Bahia and 13 from Mato Grosso do Sul proved to be the most genetically divergent. The populations from Paraná State (11 and 12) were the closest ones with 100% of bootstrap support.

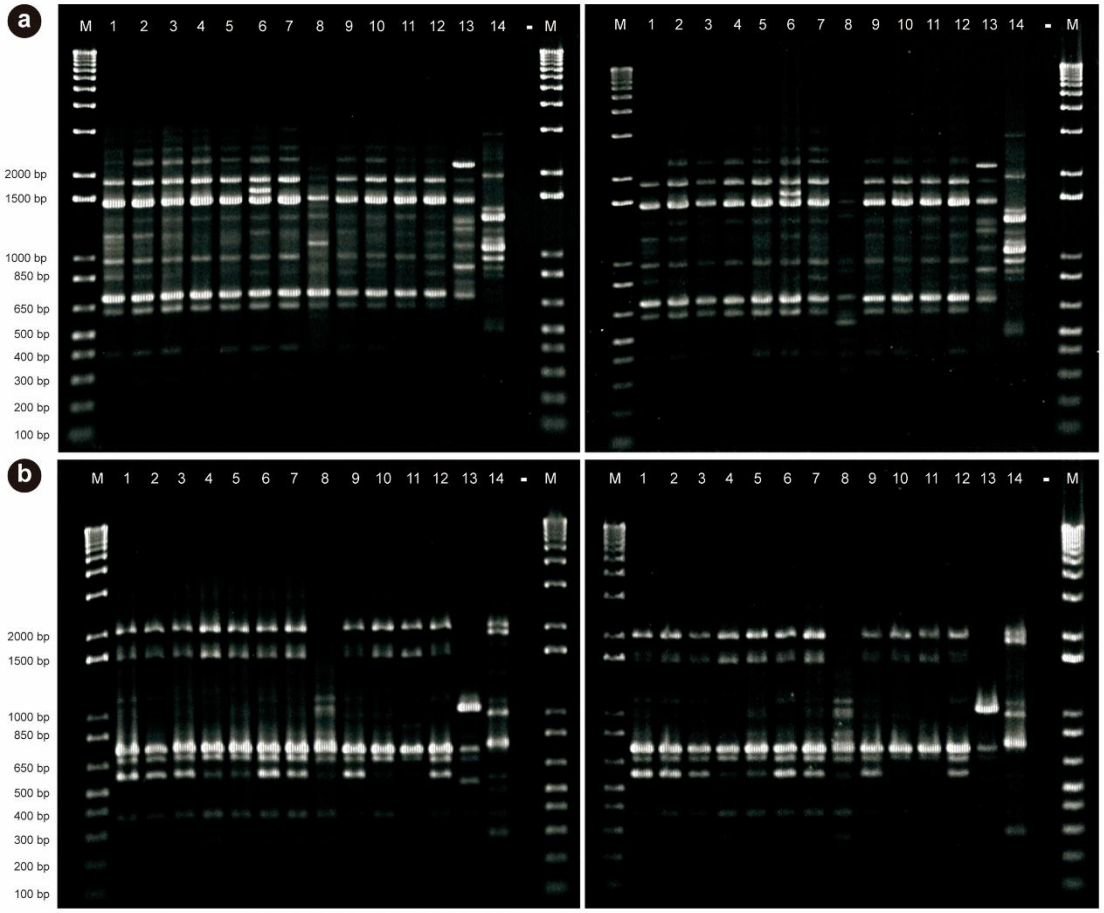

Figure 4. Genetic diversity of *Meloidogyne incognita* analyzed with primers RAPD Z4 **(a)** and AFLP 18 **(b)**. 1 – 10: isolates from Bahia; 11 and 12: isolates from Paraná; 13: isolate from Mato Grosso do Sul; 14: *M. enterolobii* (outgroup); (–): DNA negative control; M: 1 kb Plus DNA Ladder (Invitrogen); bp: base pairs.

Table 4. Percentage of polymorphisms detected in *Meloidogyne incognita** at populations level.

M. incognita populations	RAPD fragments		AFLP fragments		RAPD + AFLP fragments	
	Amplified	Polymorphic (%)	Amplified	Polymorphic (%)	Amplified	Polymorphic (%)
1 – 10; 11;12;13	621	289 (46.5)	199	72 (36.1)	820	361 (44)
1 – 10 (excluded 8); 11; 12; 13	603	235 (38.9)	185	33 (17.8)	788	268 (34)
1 – 10	590	217 (36.7)	182	43(23.6)	772	260 (33.7)
1 – 10 (excluded 8)	583	172 (29.5)	182	22 (12)	765	194 (25)

*Population data in Table 2

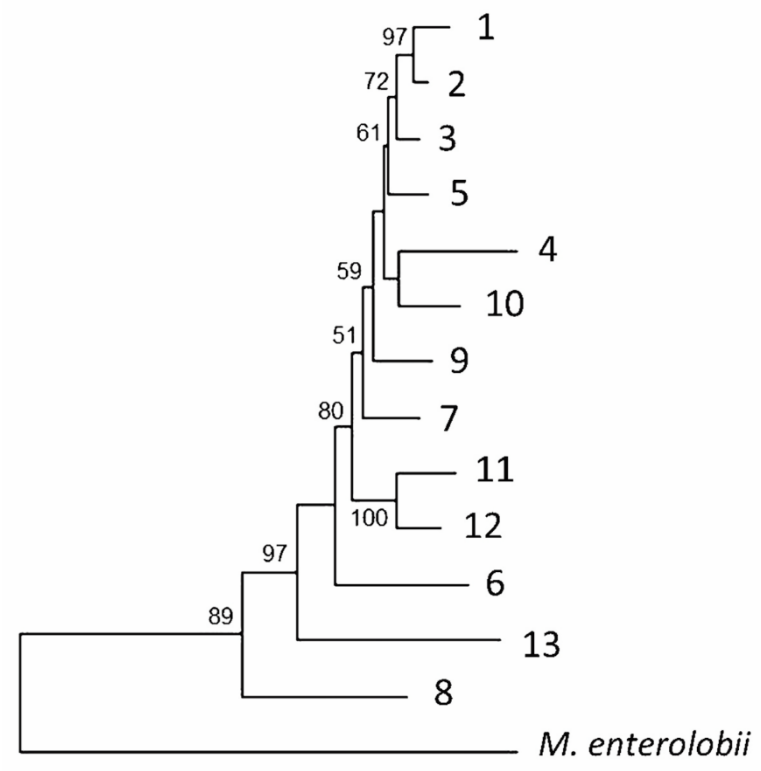

Figure 5. Phylogenetic tree of *Meloidogyne incognita* populations from Bahia (1 – 10); Paraná (11 and 12) and Mato Grosso do Sul states (13) from RAPD and AFLP markers.

3.3 Aggressiveness/Virulence of *M. incognita* populations on cotton accessions.

Aggressiveness and virulence were evaluated using the criteria of resistance and susceptibility: galling index, egg mass index, number of eggs/g of roots and reproduction factors (RF). All nematode populations tested showed reduced reproduction factors (RF<0.7) on the resistant accessions M-315 RNR and CIR1348 (with two resistance QTLs) (Table 5). Gall and egg mass formation were also partially suppressed on these cotton accessions (Table 6). The other three cultivars (Wild Mexican Jack Jones, LA 887 and Clevewilt 6) with a single resistance gene (moderate resistance) were also resistant using Hussey & Janssen (2002) concept, which predicts for the resistant accession reproduction <10% of the susceptible accession. Considering

that, none studied population from the State of Bahia was virulent to the five cotton cultivars with different resistance genes (Tables 5, 6 and 7). In contrast, the susceptible control FM966 exhibited high gall and egg mass numbers, number of eggs/g of roots and high RF for all populations (Tables 5, 6 and 7), with two of the populations (8 and 10) standing out from the others, as highly aggressive, reaching a RF of 539.3 and 218.0 (means), respectively. Comparing these results, with the analysis of genetic variability (Figure 4), the most aggressive population (8) was also the most genetically divergent for the RAPD and AFLP markers.

A correlation analysis between the evaluation parameters was performed using Pearson coefficient (Table 8). There was a general significant positive correlation between the gall index (GI), egg masses index (EMI), reproduction factor (RF) and eggs/g of roots; but for the resistant accession CIR1348 and M315 there was a low correlation between GI/EMI, GI/eggs/g of roots, GI/RF, EMI/egg/g of root, EMI/RF. In the accessions LA887, Clevewilt and WMJJ it was observed a greater correlation between EMI/RF than between GI/FR, and for all accessions RF/egg/g of root had the highest correlation.

Table 5. Reproduction Factor (RF) of six populations of *Meloidogyne incognita* from Bahia State, in cotton accessions with different levels of resistance.

Cotton accessions[a]	Population[b] 1	Population 4	Population 6	Population 7	Population 8	Population 10
FM 966	45.2 a	67.9 a	84.5 a	74.8 a	539.3 a	218 a
Wild Mexican Jack Jones	1.0 b	2.7 b	4.0 b	5.0 b	2.6 b	4.1 b
LA 887	0.3 b	1.0 c	0.8 c	1.8 c	1.4 b	0.8 c
Clevewilt 6	0.3 b	0.1 d	0.1 d	0.1 d	1.3 b	7.2 b
CIR 1348	0.1 b	0.0 d	0.7 c	0.1 d	0.1 c	0.7 c
M 315	0.0 b	0.1 d	0.0 d	0.0 d	0.0 c	0.0 d

Coefficient of variation (%) = 40.7
a- Cotton accessions data described in Table 3.

b- *M. incognita* populations data described in Table 2.
Mean values (7 plants per accession) were transformed as log (x+1). Means followed by different letters within columns are significantly different (P<0.05) according to Scott-Knot's test.

Table 6. Mean galling index (GI) and egg mass index (EMI) of six populations of *Meloidogyne incognita* from Bahia State on selected cotton accessions.

Cotton accessions[a]	Population[b] 1		Population 4		Population 6		Population 7		Population 8		Population 10	
	GI[c]	EMI[c]	GI	EMI	GI	EMI	GI	EMI	GI	EMI	GI	EMI
FM 966	5	5	5	5	5	5	5	5	5	5	5	5
Wild Mexican Jack Jones	1.8	3.6	3.8	4.5	2	4.5	4.5	3.5	3.6	3	3.8	3.2
LA 887	3.3	4.7	4	4.2	4.8	4.4	4.4	2.8	4.7	2.7	4	1.4
Clevewilt 6	0.7	1.6	1.6	1.2	1.3	4.7	4.7	0.4	1.6	0.4	1.6	0.4
CIR 1348	0.1	0.8	1.3	0	0.8	0.4	0.4	0	0.8	0	1.3	1.3
M 315	0	0.4	0	0.3	0	0.1	0.1	0	0.4	0	0	0

a- Cotton accessions described in Table 3
b- *M. incognita* populations data described in Table 2
c- Mean values (7 plants per accession) of GI or EMI. 0: no gall or egg-mass, 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)

Table 7. Eggs/(g) roots of six populations of *Meloidogyne incognita* from Bahia State, in cotton accessions with different levels of resistance.

Cotton accessions[a]	Population[b] 1	Population 4	Population 6	Population 7	Population 8	Population 10
FM 966	3844.9 a	5264 a	7494.5 a	4985.5 a	33104.5 a	13563.4 a
Wild Mexican Jack Jones	52.7 b	156.1 b	236.4 b	270.8 b	82.1 b	203.1 c
LA 887	19.1 b	46.9 b	47.7 c	100.6 b	75 b	57 d
Clevewilt 6	12.1 b	2.8 c	4.1 d	2.9 c	35.4 b	560.8 b
CIR 1348	3.9 c	0.8 c	35.7 c	6.9 c	4.8 c	38.7 d
M 315	1.5 c	2.8 c	1.1 d	0.0 d	0.7 c	0.4 e

Coefficient of variation (%) = 29.7
a- Cotton accessions data described in Table 3.
b- *M. incognita* populations data described in Table2.

Mean values (7 plants per accession) were transformed as log (x+1). Means followed by different letters within columns are significantly different (P<0.05) according to Scott-Knot's test.

Table 8. Correlation coefficients between the evaluation parameters for the six cotton accessions tested.

Cotton accessions[a]	GI/EMI	GI/Egg/g of root	GI/RF	EMI/ Egg/g of root	EMI/RF	RF/ Egg/g of root
LA 887	**0.6719** <0.0001	**0.3768** 0.0139	**0.3994** 0.0088	**0.6081** <0.0001	**0.6416** <0.0001	**0.9693** <0.0001
Clevewilt 6	**0.8792** <0.0001	**0.6547** <0.0001	**0.6795** <0.0001	**0.7261** <0.0001	**0.7645** <0.0001	**0.9869** <0.0001
Wild Mexican Jack Jones	**0.8054** <0.0001	**0.5501** 0.0002	**0.5123** 0.0005	**0.7428** <0.0001	**0.7729** <0.0001	**0.9799** <0.0001
CIR 1348	**0.3804** 0.0130	**0.2524** 0.1068	**0.1925** 0.2220	**0.2276** 0.3862	**0.0524** 0.2419	**0.9727** <0.0001
M 315	=	**0.4523** 0.0028	**0.4504** 0.0026	=	=	**0.8834** <0.0001
FM 966	=	=	=	=	=	**0.9082** <0.0001

GI- Gall index; EMI- Egg masses index; RF- Reproduction factor.
a- Cotton accessions data described in Table 3.
Pearson coefficient |r| under H0: Rho=0
Numbers in bold – correlation coefficient, numbers below – significance level, (=) standard deviation = 0.

4. DISCUSSION

This study evaluated the genetic variability and aggressiveness/virulence of 10 populations of *M. incognita* from Bahia State, Brazil and three populations from Paraná and Mato Grosso do Sul states, previously studied by Silva *et al*. (2014). Six populations from Bahia State were evaluated on different cotton cultivars that harbour resistance genes to RKN. Similar results have been reported for *M. incognita* on cotton

from different Brazilian States (Silva *et al*., 2014) and for different populations from different crops (Santos *et al*., 2012). Despite the existence of three esterase profiles for *M. incognita* (EST I1, I2 and S2) and a low genetic variability reported by Santos *et al*. (2012), only one phenotype (EST I2) was detected in all the populations, but high genetic diversity (44%) was found, mainly due to population 8, which differed significantly from the others. Removing this population from the analysis of variability, the genetic diversity was only 25%. *Meloidogyne incognita* is known to have low genetic variability due its parthenogenetic reproduction and similarities in the chromosome number (Santos *et al*., 2012). Phylogenetically, all *M. incognita* Brazilian populations clustered together with 89% bootstrap support. In addition, the populations from Paraná remained together with 100% bootstrap support, but no other geographical relationship among populations of *M. incognita* from cotton was found. Similar results were also reported for other *M. incognita* isolates (Randig *et al*., 2002; Carneiro *et al*., 2004; Santos *et al*., 2012; Silva *et al*., 2014).

The identification of races in RKN is important not only for the characterization of resistance, but also for the development of management programs in infested areas (Fassuliotis, 1985). The prevalence of race 3 in relation to race 4 in cotton was reported for the first time by Silva *et al*. (2014), and the present results confirm that RKN populations from cotton, virulent to resistant tobacco NC 95 (race 4), are less frequent. Despite the existence of two races in these populations, which is important for the establishment of management strategies, Moens *et al*. (2009) recommended the discontinuation of this terminology. Indeed, this concept has never been universally accepted because it measures a very restricted portion of the potential variation in parasitic variability. In the present study, no relationship was observed among host races and genetic polymorphism or phylogeny. These findings suggest that race 4

(population 7) has low genetic polymorphism, which is in agreement with previous observations (Carneiro & Cofcewicz, 2008). Recently, it was shown that the genomes of apomictic *Meloidogyne* are made of duplicated regions, with functional divergence between gene copies, and which are rich in transposable elements, which might be responsible for their genomic plasticity and adaptation to the environment (Blanc-Mathieu *et al*., 2017).

Population 8 was the most divergent and also highly aggressive to the susceptible cotton FM 966. This correlation between aggressiveness and genetic variability was not observed in the other populations, as another aggressive population (10) did not present high genetic divergence. Previous studies have also failed to establish this correlation (Silva *et al*., 2014; Mattos *et al*., 2016). Aggressiveness reflects the ability of nematodes to reproduce on a susceptible host, as measured by the RF, whereas virulence is their ability to reproduce on resistant hosts (Hussey & Janssen, 2002). Therefore, in this study, no *M. incognita* populations were virulent for cotton cultivars bearing resistance genes. According to Castagnone-Sereno (2002), genomic polymorphisms are independent of virulence, and are probably the result of independent mutational events.

All cotton accessions were resistant to the populations of *M. incognita* from Bahia, according to the concepts of Hussey & Janssen (2002), where RF below 10% in relation to the susceptible control is considered resistant. These results demonstrate a strong effect of available resistance genes against populations prevalent on cotton farms in Bahia. The presence of one QTL associated with RKN resistance (WMJJ, Clevewilt and LA-887) is sufficient to drastically reduce the nematode population, whereas the combination of two QTL (CIR1348 and M315) leads almost to immunity, even considering highly aggressive populations (8 and 10). This information corroborates the

possibility of using available elite strains possessing only one resistance QTL in the management of *M. incognita* in the west of Bahia in the short term. However, although there was a high population reduction, it is important that accessions with only one resistance gene allow low reproduction of the nematode. Unrestricted use of this in the long term can lead to the emergence of virulent populations. Resistance based on a few genes may impose a selection pressure on nematode populations and hasten the selection of virulent isolates, as has been observed in other crops (Janssen *et al*., 1990). Parthenogenetic species of RKN have a highly adaptive responsiveness to the environment, and their ability to overcome resistance genes has been demonstrated (Roberts, 1995; Castagnone-Sereno, 2002).

A low correlation was found between the number of eggs per g of roots and the GI, showing that, depending on the cultivar, the values may not be correlated. This means that the assessment of cultivars regarding resistance to RKN based only on GI can lead to errors, which can be explained by the fact that in several cases the resistance response is late, allowing gall development but preventing the formation of egg masses (Mota *et al*., 2013). The findings here reinforce the need to use combined parameters to evaluate the RKN resistance.

The high level of resistance to *M. incognita* found in the cotton breeding line M-315 RNR and in other lines derived from the same source (Auburn 634 RNR), has been transferred to few superior varieties. This resistance is inherited from two major genes, presumably one from Clevewilt-6 and the other from Wild Mexico Jack Jones (McPherson *et al*., 2004; Starr *et al*., 2010). Clevewilt-6 has one recessive resistance gene that confers moderate resistance to *M. incognita* (McPherson *et al*., 2004), and it is also believed to be the source of resistance in LA-887 (Jones *et al*., 1990). The same resistance allele is present in some of the cultivars in Brazil (P. Barroso, personal

communication), pointing to the need for more efficient resistance gene combinations. All the populations tested were avirulent to M-315 RNR, and all these harbour a second gene in addition to that originating from Clevewilt-6. The resistance present in Wild Mexican Jack Jones has been recently deployed in one varieti(IMA 5801 B2RF) in Brazil. This accession showed a high level of resistance to all populations, even to the most aggressive ones. The other accessions that showed high levels of resistance to all populations in this study were LA-887 and CIR1348, and they also constitute potential sources of resistance that have, to the best of the authors' knowledge, never been deployed in commercial cultivars in Brazil.

In the present study, it has been shown that these sources of resistance could have a large adaptability. Further studies are underway to find out whether the resistance gene(s) and allele(s) in LA-887 and CIR1348 are different from those present in Auburn 634 RNR and in the derived germplasm. The characterization of new *M. incognita* populations from Bahia and identification of novel sources of resistance that can be pyramided and/or rotated is an important goal towards the effective and durable management of RKN on cotton farms.

REFERENCES

Blanc-Mathieu R, Perfus-Barbeoch L, Aury JM, Da Rocha M, Gouzy J, Sallet E, Martin-Jimenez C, Bailly-Bechet M, Castagnone-Sereno P, Flot JF, Kozlowski DK, 2017. Hybridization and polyploidy enable genomic plasticity without sex in the most devastating plant-parasitic nematodes. *PLoSGenet* **13** (6).

Carneiro RMDG, 2015. Raças de *Meloidogyne* spp.: conceitos e pesquisas futuras. Anais. XXXII Congresso Brasileiro de Nematologia, 36.

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

Carneiro RMDG, Cofcewicz ET, 2008. Taxonomy of coffee-parasitic root-knot nematodes, *Meloidogyne* spp. In: Souza RM, ed. *Plant parasitic nematodes of coffee*. New York, USA: APS Press & Springer, 87-122.

Carneiro RMDG, Dos Santos MFA, Almeida MRA, Mota FC, Gomes ACMM, Tigano MS, 2008. Diversity of *Meloidogyne arenaria* using morphological, cytological and molecular approaches. *Nematology* 10, 819 – 834.

Carneiro RMDG, Tigano MS, Randig O, Almeida MRA, Sarah JL, 2004. Identification and genetic diversity of *Meloidogyne* spp. (Tylenchida: Meloidogynidae) on coffee from Brazil, Central America and Hawaii. *Nematology* **6**, 287-298.

Castagnone-Sereno P, 2002. Genetic variability of nematodes: a threat to the durability of plant resistance genes? *Euphytica* **124**, 193–199.

CONAB - Companhia Nacional de Abastecimento, 2018. Acompanhamento da safra brasileira de grãos. Oitavo levantamento de safra 2017/18, *Conab* **3,** 1-145.

Fassuliotis G, 1985. The role of the nematologists in the development of resistant cultivars. In: Sasser JN, Carter CC, eds. *An advanced treatise on Meloidogyne* Raleigh: North Carolina State University Graphics **1,** 233–240.

Galbieri R, Inomoto MM, Silva RA, 2015. Manejo de nematoides na cultura do algodoeiro em Mato Grosso. In: Belot JL, ed. *Manual de boas práticas de manejo do algodoeiro em Mato Grosso.*Cuiabá: IMAMT **2**, 214-225.

Hartman RM, Sasser JN, 1985. Identification of *Meloidogyne* species on the basis of differential host test and perineal pattern morphology. In: Barker KR, Carter CC, Sasser JN, eds. *An advanced treatise on Meloidogyne*. Methodology. Raleigh, NC, USA, North Carolina State University Graphics **2**, 69-77.

He Y, Kumar P, Shen X, Davis RF, Van Becelaere G, May OL, Nichols RL, Chee PW, 2014. Re-evaluation of the inheritance for root-knot nematode resistance in the Upland cotton germplasm line M-120 RNR revealed two epistatic QTLs conferring resistance. *Theoretical and Applied Genetics* **127**, 1343-1351.

Hussey RS, Barker KR, 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. *Plant Disease Reports* **57**, 1025–1028.

Hussey RS, Janssen GJW, 2002. Root-knot nematodes: *Meloidogyne* species. In: Starr JL, Cook R, Bridge J, eds. *Plant Resistance to Parasitic Nematodes*. CAB International, Wallingford, 43-70.

Inomoto MM, 2001. Algodão: atacado por nematoides. *Revista Cultivar* **3**, 5-7.

Janssen R, Barker J, Gommers FJ, 1990. Selection of virulent and avirulent lines of *Globodera rostochiensis* for the H1 resistance gene in *Solanum tuberosum* spp. *andigena* CPC 1673. *Revue de Nematologie***13**, 265.

Jenkins JN, Mccarty JC, Wubben MJ, Hayes R, Gutierrezo A, Callaha F, Deng D, 2012. SSR markers for marker assisted selection of root-knot nematode (*Meloidogyne incognita*) resistant plants in cotton (*Gossypium hirsutum* L). *Euphytica* **183**, 49-54.

Jones JE, Dickson JL, Aguillar W, Caldwell WD, More SH, Hutchinson, RI, 1990. Stoneville LA 887: a new cotton variety. *Louisiana Agriculture* **33**, 5.

Mattos VS, Furlanetto C, Silva JGP, Santos DF, Almeida MRA, Correa VR, Moita AW, Castagnone-Sereno P, Carneiro RMDG, 2016. *Meloidogyne* spp. populations from native Cerrado and soybean cultivated areas: genetic variability and aggressiveness. *Nematology* **18**, 1-11.

McPherson MG, Jenkins JN, Watson CE, Mccarty JC, 2004. Inheritance of root-knot nematode resistance in M-315 RNR and M78-RNR cotton. *Journal of Cotton Science* **8**, 154–161.

Moens M, Perry RN, Starr JL, 2009. *Meloidogyne* species – a diverse group of novel and important plant parasites. In: Perry RN, Moens M, Starr JL, eds. *Root-knot nematodes*.CABI International, Wallingford, 1-17.

Mota FC, Alves GCS, Giband M, Gomes ACMM, Sousa FR, Mattos VS, Barbosa VHS, Barroso PAV, Michel N, Peixoto JR, Rocha MR, Carneiro RMDG, 2013. New sources of resistance to *Meloidogyne incognita* race 3 in wild cotton accessions and histological characterization of the defense mechanisms. *Plant Pathology* **62,** 1173-1183.

Ogallo JL, Goodell PB, Eckert J, Roberts PA, 1997. Evaluation of Nem X, a new cultivar of cotton with high resistance to *Meloidogyne incognita*. *Journal of Nematology* **29**, 531–537.

Perina FJ, Bogiani JC, Ribeiro GC, Breda CE, Fabris A, Santos IA, 2017. Levantamento e manejo de fitonematoides em algodoeiro no oeste da Bahia, resultados safra 2016/17. *Circular Técnica Fundação-BA* **3**, 1-6.

Randig O, Bongiovanni M, Carneiro RMDG, Castagnone-Sereno P, 2002. Genetic diversity of root-knot nematodes from Brazil and development of SCAR markers specific for the coffee-damaging species. *Genome* **45**, 862-870.

Roberts PA, 1995. Conceptual and practical aspects of variability in root-knot nematodes related to host plant resistance. *Annual Review of Phytopathology* **33**, 199-221.

Santos MFA, Furlanetto C, Carneiro MDG, Almeida MRA, Mota FC, Gomes ACMM, Silveira NOR, Silva JGP, Castagnone-Sereno P, Tigano MS, Carneiro RMDG, 2012. Biometrical, biological, biochemical and molecular characteristics of *Meloidogyne incognita* isolates and related species. *European Journal of Plant Pathology* **134**, 671-684.

Semblat JP, Wajnberg E, Dalmasso A, Abad P, Castagnone-Sereno P, 1998. High-resolution DNA fingerprinting of parthenogenetic root-knot nematodes using AFLP analysis. *Molecular Ecology* **7**, 119-125.

Shepherd RL, 1974. Transgressive segregation for root-knot nematode resistance in cotton. *Crop Science* **14**, 872-875.

Silva EH, Mattos VS, Furlanetto C, Giband M, Barroso PAV, Moita AW, Jorge-Junior A, Correa VR, Castagnone-Sereno P, Carneiro RMDG, 2014. Genetic variability and virulence of *Meloidogyne incognita* populations from Brazil to resistant cotton genotypes. *European Journal of Plant Pathology* **139**, 195-204.

Starr JL, Moresco ER, Smith CW, Nichols RL, Roberts PA, Chee P, 2010. Inheritance of resistance to *Meloidogyne incognita* in primitive cotton accessions from Mexico. *Journal of Nematology* **42**, 352–358.

Suazo A, Hall HG, 1999. Modification of the AFLP protocol applied to honey bee (*Apis mellifera* L.) DNA. *BioTechniques* **26**, 704–709.

Swofford DL, 2002. PAUP* Phylogenetic analysis using parsimony (*and other methods). Sinauer Associates, Sunderland.

Trudgill DL, Blok VC, 2001. Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. *Annual Review of Phytopathology* **39**, 53-77.

Wang C, Roberts PA, 2006. A *Fusarium* wilt resistance gene in *Gossypium barbadense* and its effect on root-knot nematode–wilt disease complex. *Phytopathology* **96**, 727–34.

Zhou E, Wheeler TA, Starr JL, 2000. Root galling and reproduction of *Meloidogyne incognita* isolates from Texas on resistant cotton genotypes. *Journal of Nematology* **32**, 513–518.

Ziljstra C, Donkers-Venne DTHM, Fargette M, 2000. Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterized amplified regions (SCAR) based PCR assays. *Nematology* **2**, 847-853.

CHAPTER 3

Marker assisted selection for resistance in *Gossypium* genotypes to *Meloidoyne incognita*.

ABSTRACT

The root knot nematode *Meloidogyne incognita* is one of the main parasites causing economic losses in the cotton crop. Many efforts have been done to control this pathogen however, the most desired control strategy is the genetic resistance. Sources of resistance in cotton have been known for several years but only few commercial varieties have been released. The cotton breeding program carried out by EMBRAPA has developed lines with resistance genes from two different sources, M-315 and CIR 1348, using marker assisted selection. In order to validate the molecular markers associated with the resistance genes, nematode phenotyping was carried out in controlled environment and confirmed by genotyping. The markers originated from the M-315 resistance source were highly efficient in the selection of plants resistant to *M. incognita*, with 100% of the evaluated plants expressing a reproduction factor inferior to 0,08. Although, the CIR 1348 resistance source markers were also very efficient in selecting resistance, some segregation events revealed the need to look for markers closer to the resistance QTLs.

Key-words: Root-knot nematode; cotton; resistance genes.

RESUMO

Seleção assistida por marcadores para resistência em genótipos de *Gossypium* a *Meloidoyne incognita*.

O nematoide das galhas *Meloidogyne incognita* é um dos principais parasitas causadores de perdas econômicas na cultura do algodão. Muitos esforços têm sido feitos para controlar este patógeno, porém a estratégia de controle mais desejada é a resistência genética. Fontes de resistência no algodão são conhecidas há vários anos, mas poucas variedades comerciais foram liberadas. O programa de melhoramento de algodão realizado pela EMBRAPA desenvolveu linhagens com genes de resistência de duas fontes diferentes, M-315 e CIR 1348, utilizando seleção assistida por marcadores moleculares. Para validar os marcadores moleculares associados aos genes de resistência, a fenotipagem com o nematoide foi realizada em ambiente controlado e confirmada por genotipagem. Os marcadores originários da fonte de resistência M-315 foram altamente eficientes na seleção de plantas resistentes a *M. incognita*, com 100% das plantas avaliadas expressando um fator de reprodução inferior a 0,08. Embora os marcadores de fonte de resistência CIR 1348 também tenham sido muito eficientes na seleção de resistência, alguns eventos de segregação revelaram a necessidade de buscar marcadores mais próximos dos QTLs de resistência.

Palavras-chave: Nematoide das galhas radiculares; algodão; genes de resistência.

1. INTRODUCTION

Gossypium hirsutum L. *latifolium* Hutch, referred as Upland cotton, accounts for over 90% of world production (Jenkins, 2003). *Gossypium barbadense*, commonly

termed Pima, Sea Island, Egyptian, or extra-long fiber, represents approximately 5% of world fiber production (Wu *et al*., 2005). Upland cotton has been intensively cultivated in Brazilian Cerrado since early 1980´s and, nowadays, more than 90% of cotton growing areas are in this region (Silva Neto *et al*., 2016). Cotton supply chain contributed in 2017 with U$ 74 billion to Gross Domestic Product (GDP) and is responsible for generating over 1.3 million direct jobs (ABRAPA, 2017). Recent genetic advances along with a better cropping system, allowing high fiber yields and quality, ensuring international competitiveness of Brazilian cotton business (Morello *et al*., 2015; Barroso *et al*., 2017; Suassuna *et al*., 2017). However, this tropical region is subject to high biotic stress pressure, and currently requires extensive pesticides inputs to achieve high levels of production. The root-knot nematode [*Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949] is a serious pathogen, of increasing importance throughout cotton-growing regions (Galbieri and Asmus, 2016).

Host resistance is the major focus of most crop disease management strategies. Genetic resistance is the most desired disease control measure by the farmers, as it promotes a reduction of the pathogen populations at the same time as it allows cultivation of the crop of interest (Weaver, 2015). However, few low-yielding 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 strains in cotton breeding programs. This difficulty can be overcome by the use of molecular markers linked to resistance QTLs (Suassuna *et al*., 2016). Implementation of the use of markers assisted selection (MAS) in routine, allows to select genotypes carrying desirable alleles and to advance generations of cross, performing nematode phenotyping only in the more advanced stages of the program, with limited number of lines (Yuksel *et al*., 2016).

Currently two robust sources of resistance are available for cotton breeding programs in Brazil. The first was originated from Auburn 623 RNR, line available for more than 40 years (Shepherd, 1974). The high level resistance found in Auburn 623 RNR has been transferred by backcrossing method to give rise the Auburn 634 RNR and several M-lines. These lines have been used by different cotton breeding programs (Robinson, 2008), mainly M-120, M-240 and M-315. Genetic resistance in M-lines appears to be oligogenic inherited, determined by at least two QTLs located on chromosomes 11 and 14. Auburn 623 RNR was originated from a transgressive segregating derived from the crossing between two moderately resistant accessions, Clevewilt 6 and Wild Mexican Jack Jones (WMJJ). The QTL (*qMi-C11*), originating from Clevewilt 6, has a dominant gene effect affecting gall formation and is located in the interval CIR069-CIR316 on chromosome 11. Whereas QTL (*qMi-C14*), originating from WMJJ, has partial dominant gene effect, and is associated with reduced egg production and is in the interval BNL3545 - BNL3661 on chromosome 14. An epistatic interaction between the two genes confers a near-immunity resistance to the RKN in the genotypes carrying both genes which could not be explained only by an additive effect of the two genes individually (Mcpherson *et al*., 2004; Shen *et al*., 2006, 2010; Ynturi *et al*., 2006; Gutiérrez *et al*., 2010; Jenkins *et al*., 2012; He *et al*., 2014).

The second source of resistance, the accession CIR1348 (*Gossypium barbadense*), was recently described (Mota *et al*., 2013; Silva *et al*., 2014). In contrast to Auburn 623 RNR line, resistance in CIR1348 has partially recessive oligogenic inheritance (Gomez *et al*., 2016). The genetic mapping identified two major effect QTLs on chromosome 11 and chromosome 15, which are responsible for the high level of resistance to the nematode (Silva *et al*., 2014; Gomez *et al*., 2016). The association between markers (CIR069, CIR316 and SHIN1425) and QTL of nematode resistance on

chromosome 11 and markers (JESPR152 and NAU3254) on resistance QTL on chromosome 15 were confirmed (Gomez *et al*., 2016; Silva *et al*, 2014). The association of molecular markers with resistance genes has allowed the routinely use of MAS strategy in EMBRAPA Cotton breeding program. Several segregating populations have been generated, and MAS were used in early generations plant selection and a series of elite lines have been obtained using M-315 and CIR 1348 sources of resistance (Suassuna *et al*., 2019).

In this study, we report phenotypic and genotypic data from RKN resistant cotton lines (early generation selected using MAS) to confirm and validate the efficiency of SSR markers linked to resistance genes.

2. MATERIALS AND METHODS

2.1 Cotton germplasm

*Lines derived from M-315***:** Segregating population was generated from a triple cross [(BRS 368RF x M-315) x 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. Population was advanced in bulk for three generations. F4 plants were selected and genotyped to CBD, BB, and RKN resistance using SSR molecular marker DC20027 (Fang *et al*., 2010), CIR246 (Xiao *et al*., 2010), CIR 316 and BNL 3661 (Gutiérrez *et al*., 2010). All plants that amplified the homozygous band pattern associated with all resistance genes (76 in total) were selected to progeny row tests. In 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 CIR 1348***:** A cross and two subsequent backcrosses were performed using *G. barbadense* CIR 1348 as RKN resistance donor parent and *G.*

hirsutum cultivar Fibermax 966 as a recurrent parental. BC_2F_2 plants were genotyped using SSR markers CIR 069, CIR 316, SHIN 1425, JESPR 152 and NAU 3254. All plants that amplified the homozygous band pattern associated with resistance QTLs were selected to progeny row tests in net house. BC_2F_4 individual plants were selected in progeny rows and give rise to the lines used in this study.

Divergent accessions: It was also included three divergent cotton germplasm: a *G. hirsutum* L. 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, however not carrying any known SSR markers, CNPA GO 2002-2043/5. Details on accessions are in Table 9. These lines had not been evaluated previously, except CNPAGO 2002-2043/5). *Gossypium hirsutum* cv. FiberMax 966 (FM966) was used as a susceptible control, while *G. hirsutum* line M-315 RNR was used as a resistant control.

Table 9. Accessions of *Gossypium* spp. used in the study of marker assisted selection of *Gossypium* spp. for resistance to *Meloidogyne incognita*

Cotton accessions	Species	Genealogy	Comments
FM966	*G. hirsutum*	(DP90 x 75007-3) x (DP90 x Tamcot SP37H)	Commercial variety susceptible to RKN
CNPA5M	*G. hirsutum* var. *marie-galante*	Recurrent selection in CNPA 3M	Obsolete cotton cultivar, supposedly tolerant do RKN
CNPA2015-1800FL	*G. barbadense*	Pima S-7 x Pima S-6	Allele 185/189 to BLN 3661marker
CNPAGO2002-2043/5	*G. hirsutum*	BRS Aroeira x (VAP 206 x Delta Opal)	Partial resistant line without known resistance alleles
CNPAT164-5	*G. barbadense* x *G.hirsutum*	([CIR1348xFM966] x FM 966) x FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPAT150-11	*G. barbadense* x *G.hirsutum*	([CIR1348 x FM 966] x FM 966) x FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPAT60-8	*G. barbadense* x *G.hirsutum*	([CIR1348 x FM 966] x FM 966) x FM 966	RC_2F_2 homozygous plants to CIR1348 markers

CNPAT143-1	*G. barbadense* x *G.hirsutum*	([CIR1348 x FM 966] x FM 966) x FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPAT60-1	*G. barbadense* x *G.hirsutum*	([CIR1348 x FM 966] x FM 966) x FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPAT60-4	*G. barbadense* x *G.hirsutum*	([CIR1348 x FM 966] x FM 966) x FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPAT109-14	*G. barbadense* x *G.hirsutum*	([CIR1348 x FM 966] x FM 966) x FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPAT104-6	*G. barbadense* x *G.hirsutum*	([CIR1348 x FM 966] x FM 966) x FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPAT3-6	*G. barbadense* x *G.hirsutum*	([CIR1348 x FM 966] x FM 966) x FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPAT73-1	*G. barbadense* x *G.hirsutum*	([CIR1348 x FM 966] x FM 966) x FM 966	RC_2F_2 homozygous plants to CIR1348 markers
CNPA17-17 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA17-40 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-15 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-58 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-50 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-28 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-21 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-26 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-13 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-22 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-35 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-49 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-53 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-55 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-12 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers

CNPA 17-18 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-33 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-34 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
CNPA 17-56 B2RF	*G. hirsutum*	(BRS 368 RFxM-315) x BRS 430B2RF	Homozygous line to M-315 markers
M-315		USA – *G. hirsutum*	Breeding line with the RKN resistance QTL *qMi-C11* and *qMi-C14*

2.2 Nematode inoculum

It was used a pool of 15 *M. incognita* populations collected in infested areas in Brazil 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 and a blender instead of manual agitation (Hussey & Barker, 1973). The counting was done using a light microscope and Peter's slides.

2.3 RKN resistance in cotton germplasm

Eight plants of each germplasm 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 Floxin B and evaluated for gall and egg mass indexes, 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 & Sasser, 1985). Eggs were extracted according to Hussey & Barker (1973) methodology, using a blender instead of manual agitation and 1 % NaOCl. 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 ($P<0.05$).

2.4 SSR genotyping

Young leaf tissues 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 BNL 3661 and CIR 316, and lines derived from the source of resistance CIR-1348 were also genotyped with markers CIR 069, SHIN 1425, CIR 316, JESPR 152 and NAU 3254. The PCR products were added with 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.

3. RESULTS

Resistance was evaluated based on the criteria: gall index (GI), egg mass index (IMO), and reproduction factor (RF). The results of the phenotypic evaluation are shown in Table 10. The susceptible check, FM 966, exhibited high levels of gall and

egg masses indexes and had a high level of reproduction of the nematode (RF average =13). The accession CNPA5M (*G. hirsutum* L. var. *marie-galante* (Watt) Hutch.) was the most susceptible (RF average = 35) and had the greatest variation around the mean (Table 10).

Along with phenotypic evaluation, complementary genotyping of each individual single plants was performed, and genotyping results are synthetized in Table 11 and the expected alleles sizes to each marker is on table 12. In the genotyping assay, segregation was observed for the markers CIR 316 and BNL 3661 in CNPA 5M, and different alleles patterns appeared for the marker CIR 316, including alleles found in *G. barbadense*, *G. hirsutum* and one non-common allele, 192 (Table 11).

All genotypes derived from CIR 1348 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 less than 1, however, it was observed segregation 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 10).

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 10), comparable to the resistant M-315 control. Galling and reproduction differences between the susceptible control (FM 966), one line derived from M-315 *qMi-C11* and *qMi-C14* positive and line homozygous for CIR 1348 markers, after staining with fuchsin, are illustrate in figure 6.

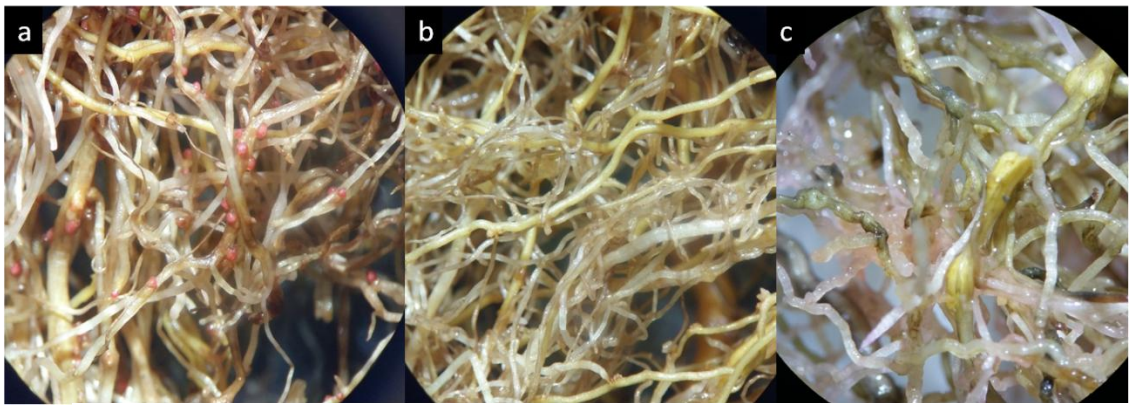

Figure 6. Cotton roots after 4 reproduction cycles of *Meloidogyne incognita*. (a)-Susceptible check FM966 exhibiting several galls and egg masses; (b)-CNPA17-26B2RF line (treatment 21), roots without any gall or egg masses; (c)- CNPA T109-14 line (treatment 10), formation of few galls but none egg mass.

Table 10. Mean phenotypic values of 34 accessions of cotton with different levels of resistance to *Meloidogyne incognita*.

Treat. Nº	Cotton accessions[a]	Reproduction Factor		Gall index		Masses index	
		Mean[b]	Range	Mean	Range	Mean	Range
SS	FM966	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
RS	M315	0.020 f	0.0 – 0.07	0.6	0 – 2	0.0	0 – 0

SP – Susceptible standard; RP – Resistant standard

a- Cotton accessions described in Table 9.

b-Mean values (8 plants per accession) are 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

Table 11. Alleles found to each marker in genotyping assay of *Gossypium* spp. lines.

Marker Origin		*Gossypium hirsutum* M-315		*Gossypium barbadense* CIR 1348			
	Chromosome	11	14	11		15	
Treat Nº	Cotton accessions[a]	CIR 316	BNL 3661	CIR 069	SHIN 1425	JESPR152	NAU 3254
SS	FM966	198/201	191/193/195	260	213	240	285
1	CNPA 5M	192/195/198/201/203	193/195				
2	CNPA 2015-1800FL	192/203	185/191	264	221	174	277
3	CNPA GO 2002-2043/5	198/201	191/193/195	264	221	174	277
4	CNPA T164-5	201/203	191/195	264	221	174/240	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	174/240	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				
RS	M315	201/210	185/191	260	213	240	291

SS – Susceptible standard; RS – Resistant standard
Numbers in columns = Alleles size (bp)

Table 12. Alleles expected to each marker in Marker Assisted Selection of *Gossypium* spp.

Marker origin	*Gossypium hirsutum* M-315		*Gossypium barbadense* CIR 1348			
Chromosome	C11	C14	C11		C15	
	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 = Alleles size (bp)

4. 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 organism, which could incur in false resistant selections. Plant genotypes tests to reaction to nematodes under controlled environment are very efficient, since the plants are inoculated and evaluated individually, which, in turn, is very laborious and makes difficult large scale evaluations. The recent knowledge on mapping and development of molecular markers associated with resistance genes and its application in breeding programs greatly facilitated the selection of resistant plants based only on genotypic analysis, leaving the phenotypic trials in greenhouse restricted only 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 remain unknown (Menezes *et al*., 2014). *Gossypium hirsutum* race *marie-galante* has great

adaptability to Brazilian semi-arid environment having cultural importance 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 a great susceptibility to the parasitism of the nematode and the genotyping test revels the presence of both *G. hirsutum* and *G. barbadense* alleles, besides an additional unknown allele at CIR 316 loci, implying in a third yet unknown origin.

Pima cotton (*G. barbadense*) is known for its superior fiber quality (length, fineness, and strength), and highly valued in the premium textile market. In a previous genotyping in accession CNPA 2015-1800FL Pima (treatment 2), the presence of allele 185 was detected for BNL 3661 marker, however the presence of this allele did not correlate with high levels of resistance in phenotyping, this demonstrate that allele 185 – BNL 3661 confers resistance only on *G. hirsutum*.

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 is associated with CIR 316 SSR marker, while the second QTL was mapped on chromosome 14, called *qMi-C14* is associated with BNL 3661 marker (Ynturi *et al*., 2006; Wang *et al*., 2006; McPherson *et al*., 2004; Gutiérrez *et al*., 2010). 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 fragment involving both QTLs and associated molecular markers were transferred to the breeding lines, once it was not possible to detect recombination events between the markers and QTLs. The 201/210 alleles from CIR 316 marker associated with *qMi-C11*,

as well as the alleles 185/191 from BNL 3661 marker (*qMi-C14*) were found in all the lines originated from M-315 resistance source and all plants selected based on these markers showed a very low RF (0.0 - 0.13), these results are in agreement with several studies using these same markers to select resistant plants from lines derived from Auburn 623 RNR (Ulloa *et al*., 2010; Jenkins *et al*., 2012; He *et al*., 2014). 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 CIR 1348 (*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 a F_2 population originated from the cross CIR1348xFM966 it was found a ratio of 1/16 resistant individuals, which corresponds to a genetic resistance model regulated by two recessive genes, corroborating the results previously described (Silva *et al*., 2011). It was demonstrated through histopathological characterization of plant-nematode interaction that the resistant genotype CIR1348 has two post infectives mechanisms of resistance (Mota *et al*., 2012). The source CIR1348 was donator of resistance to the lines CNPA T (treatments 4 to 13), the markers CIR069, CIR316 and SHIN1425 (chromosome 11), and JESPR152, NAU3254 (chromosome 15) were used to early generation MAS in a population BC_2F_2. Although, the alleles found in genotype assay in general were correlated with low values of RF, GI and EMI, one plant in each treatment 4, 5 and 6 (CNPA T164-5, CNPA T 150-11, CNPA T60-8) had high values to these variables. Possibly, the markers on chromosome 11 may have segregate in block, however, there may have been a recombination event 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 planted 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 CIR 1348 clearly need to be fine-mapped, once 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 crossing between CIR 1348 and M-315 could clarify the relation between the genes or alleles of these two sources of resistance.

REFERENCES

ABRAPA – Associação Brasileira dos Produtores de Algodão. 2017. Disponível em: <http://abrapa.com.br/>. Access in: 23 set. 2018.

Barroso PAV, Suassuna ND, Pedrosa MB, Morello CL, Silva JL, Lamas FM, Bogiani JC, 2017. BRS 368RF: A glyphosate tolerant, midseason upland cotton cultivar for Northeast and North Brazilian savanna. *Crop Breeding and Applied Biotechnology* **17**, 388-402.

Galbieri R, Asmus GL, 2016. Principais espécies de nematoides do algodoeiro no Brasil. In: Galbieri R, Belot JL. eds. *Nematoides fitoparasitas do algodoeiro nos cerrados brasileiros: biologia e medidas de controle.* Cuiabá: IMAmt, 11-36.

Gomez GM, Moretzsohn MC, Da Silva EH, Furlanetto C, Silva JGP, Lacape JM, Barroso PAV, Carneiro RMDG, Giband M, 2016. Mapping of QTLs identified in a novel source of resistance to the root-knot nematode (*Meloidogyne incognita* race 3) in

cotton (*Gossypium barbadense* L.). *Proceedings of the world cotton research conference*, Goiânia, Brazil 163-164.

Gutiérrez OA, Stelly DM, Saha S, Jenkins JN, Mccarty JC, Raska DA, Scheffler BE, 2010. SSR markers closely associated with genes for resistance to root- knot nematode on chromosomes 11 and 14 of Upland cotton. *Theoretical and Applied Genetics* **121**, 1323-1337.

Hartman KM, Sasser JN, 1985. Identification of *Meloidogyne* species on the basis of differential host test and perineal pattern morphology. In: Carter CC, Sasser JN, eds. *An Advanced Treatise on Meloidogyne*,vol. II Methodology. Raleigh NC, USA: North Carolina StateUniversity Graphics, 69–77.

He Y, Kumar P, Shen X, Davis RF, Van Becelaere G, May OL, Nichols RL, Chee PW, 2014. Re-evaluation of the inheritance for root-knot nematode resistance in the Upland cotton germplasm line M-120 RNR revealed two epistatic QTLs conferring resistance. *Theoretical and Applied Genetics* **127**, 1343-1351.

Hussey RS, Barker KR, 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. *Plant Disease Reporter* **57**, 1025–1028.

Jenkins JN, 2003. Cotton. In: Beversdof WD. ed. *Traditional crop breeding practices: an historical review to serve as a baseline for assessing the role of modern biotechnology*. OECD, Paris, 61-70.

Jenkins JN, Mccarty JC, Wubben MJ, Hayes R, Gutierrezo A, Callaha F, Deng D, 2012. SSR markers for marker assisted selection of root-knot nematode (*Meloidogyne incognita*) resistant plants in cotton (*Gossypium hirsutum* L). *Euphytica* **183**, 49-54.

McCarty JC, Jenkins JJN, Wubben MJ, Hayes RW, Callahan FE, Deng D, 2017. Registration of six germplasm lines of cotton with resistance to the root-knot and reniform nematodes. *Journal of Plant Registrations* **11**, 168–171.

McPherson MG, Jenkins JN, Watson CE, Jr MC, 2004. Inheritance of root-knot nematode resistance inM-315 RNR and M-78 RNR cotton. *Journal of Cotton Science*, **8**, 154–161.

Menezes IPP, Gaiotto FA, Suassuna ND, Hoffmann LV, Barroso PAV, 2014. Susceptibility of *Gossypium mustelinum* populations to the main cotton diseases in Brazil. *Journal of Agricultural Science* **6**, 39-47.

Morello CL, Suassuna ND, Barroso PAV, Silva JL, Ferreira ACB, Lamas FM, Pedrosa MB, Chitarra LG, Ribeiro JL, Godinho VPC, Lanza MA, 2015. BRS 369RF and BRS 370RF: Glyphosate tolerant, high-yielding upland cotton cultivars for central Brazilian savanna. *Crop Breeding and Applied Biotechnology* **15**, 290-294.

Mota FC, Alves GCS, Giband M, Gomes ACMM, Sousa FR, Mattos VS, Barbosa VHS, Barroso PAV, Michel N, Peixoto JR, Rocha MR, Carneiro RMDG, 2013. New sources of resistance to *Meloidogyne incognita* race 3 in wild cotton accessions and histological characterization of the defense mechanisms. *Plant Pathology* **62,** 1173-1183.

Robinson AF, 2008. Nematode management in cotton. In: Ciancio A, Mukerji KG. eds. *Integrated management of biocontrole of vegetable and grain crops nematodes*. Springer, 149-182.

Shen X, He Y, Lubbers EL, Davis RF, Nichols RL, Chee PW, 2010. Fine mapping QMi-C11 a major QTL controlling root-knot nematodes resistance in Upland cotton. *Theoretical and Applied Genetics* **121**, 1623-1631.

Shen X, Van Becelaere G, Kumar P, Davis RF, Chee P, 2006. QTL mapping for resistance to root-knot nematodes in the M-120 RNR Upland cotton line (*Gossypium hirsutum*L.) of the Auburn 623 RNR source. *Theoretical and Applied Genetics* **113**, 1539-1549.

Shepherd RL, 1974. Transgressive segregation for root-knot nematode resistance in cotton. *Crop Science* **14**, 872-875.

Silva EH, 2014. Variabilidade genética e fisiológica de populações de *Meloidogyne incognita* e identificação de QTLs de uma nova fonte de resistência do algodoeiro (*Gossypium* spp.) a esse nematoide. *Tese de Doutorado*. Universidade de Brasília – DF.

Silva EH, Carneiro, MDG Giband, M, Barroso PVA, Furlanetto C, Carneiro RMDG, 2011. Estudo preliminar da herança da resistência a *Meloidogyne incognita* raça 3 em algodoeiro. In: XXIX Congresso Brasileiro de Nematologia. Brasília-DF. *Anais do XXIX congresso Brasileiro de Nematologia*. Brasília - DF: Embrapa informação Tecnológica **1**, 202-203.

Silva EH, Mattos VS, Furlanetto C, Giband M, Barroso PAV, Moita AW, Jorge-Junior A, Correa VR, Castagnone-Sereno P, Carneiro RMDG, 2014. Genetic variability and virulence of *Meloidogyne incognita* populations from Brazil to resistant cotton genotypes. *European Journal of Plant Pathology* **139**, 195-204.

Silva Neto SB, Pereira AF, Morello CL, Suassuna ND, 2016. Melhoramento clássico e biotecnologia visando a superação de desafios. In: Piccoli GV, Dalcin E. eds. Desafios do cerrado: Como sustentar a expansão da produção com produtividade e competitiva. Associação Matogrossense dos Produtores de Algodão, Cuiabá, 215 – 253.

Suassuna ND, Lopes CML, Perina FJ, Silva Filho JL, Morello CL, Carneiro RMDG, 2019. Pyramiding resistance to main cotton diseases in Brazil. Beltwide Cotton Conferences, New Orleans, MS, USA. *Natl Cotton Council of America*, Memphis, TN, USA, p 39.

Suassuna ND, Morello CL, Pedrosa MB, Barroso PAV, Silva JL, Suassuna TMF, Perina FJ, Sofiatti V, Magalhães FOC, Farias FJC, 2017. BRS 430 B2RF and BRS 432 B2RF: Insect resistant and glyphosate-tolerant high-yielding cotton cultivars. *Crop Breeding and Applied Biotechnology* **18**, 221-225.

Suassuna ND, Scoz LB, Giband M, 2016. Melhoramento genético do algodoeiro para resistência aos nematoides: seleção assistida por marcadores moleculares. In: Ghalbieri R, Bellot JL. eds. *Nematoides fitoparasitas do algodoeiro nos cerrados brasileiros: biologia e medidas de controle.* Cuiabá: Instituto Mato-grossense do Algodão, 243-256.

Ulloa M, Wang C, Roberts PA, 2010. Gene action analysis by inheritance and quantitative trait loci mapping of resistance to root-knot nematodes in cotton. *Plant Breeding* **129**, 541–550.

Wang C, Ulloa M, Roberts PA, 2006. Identification and mapping of microsatellite markers linked to a root-knot nematode resistance gene (rkn1) in Acala NemX cotton (*Gossypium hirsutum* L.). *Theor Appl Genet* **112**, 770–777.

Weaver DB, 2015. Cotton nematodes. In: Fang DD, Percy RG, eds. *Cotton*, Madison, United States: ASA, CSSA and SSSA, 1-24.

Wu Z, Solimar KM, Zipf A, Saha S, Sharma GC, Jenkins JN, 2005. Isolation and characterization of genes differentially expressed in fiber of *Gossypium barbadense* L. *The Journal of Cotton Science* **9**, 166-174.

Xiao J, Fang DD, Bhatti M, Hendrix B, Cantrell RG, 2010. A SNP haplotype associated with a gene resistant to *Xanthomonas axonopodis* pv. *malvacearum* in upland cotton (*Gossypium hirsutum* L.) *Molecular Breeding* **25**, 593–602.

Ynturi P, Jenkins JN, MccartyJC, Gutierrez OA, Saha S, 2006. Association of root-knot nematode resistance genes with simple sequence repeat markers on two chromosomes in cotton. *Crop Science* **46**, 2670-2674.

Yuksel B, Khezir H, Adem B, Muhammad TA, 2016, Insight in the utilization of marker assisted selection in cotton, *Molecular Plant Breeding* **7** (10) 1-17

CHAPTER 4

Near Immunity resistance to *Meloidogyne incognita* characterize the line CNPA 17-26 B2RF of *Gossypium hirsutum*.

ABSTRACT

Meloidogyne incognita is the most important pathogenic nematode on cotton. In this work the aim was to histologically characterize the resistance of CNPA17-26B2RF line derived from the triple cross [BRS 368 RF x M-315] x BRS 430 B2RF] to *M. incognita*. This resistance was compared to FiberMax 966, a cotton variety known to be susceptible to the nematode. In greenhouse assays, the reproduction factor (RF), gall (GI) and egg-mass (EMI) index were almost zero in the resistant line and in the susceptible FM 966: RF = 13, IG=5, IMO =5. Nevertheless, *M. incognita* penetrated equally in both genotypes. In the histopatological study a strong blue fluorescence was observed around the nematode (hypersensitivity reaction, RH), mainly in the beginning (from 2-6 DAI) in the cortex and central cylinder of 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, showing also the presence of phenolic compounds involving 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 adult females were observed. Only fourth stage juveniles (J4s) and males were frequently visualized at 34 DAI. This resistance mechanism characterizes almost immunity, no females and no eggs production. In susceptible control it was possible to

visualize feeding sites well developed from 6 to 30 DAI. Females reached maturity at 26 DAI and egg production at 30 DAI. Our results suggested that the resistance (near immunity) of the line CNPA17-26B2RF was related to early (2-12 DAI) defense responses that prevented totally the nematode reproduction.

Key words: plant resistance; hypersensitive response; root-knot nematode.

RESUMO

Quase imunidade contra *Meloidogyne incognita* caracteriza a Linhagem CNPA17-26 B2RF de *Gossypium hirsutum* .

Meloidogyne incognita é o mais importante nematoide patogênico do algodão. Neste trabalho, objetivou-se caracterizar histologicamente a resistência da linhagem CNPA17-26B2RF derivada do cruzamento triplo [BRS 368 RF x M-315] x BRS 430 B2RF]. A resistência dessa linhagem foi confirmada quando comparada à cultivar FiberMax966, conhecida por ser suscetível ao nematoide. Nos ensaios em casa de vegetação, o fator de reprodução (FR), índice de galhas (IG) e índice de massas de ovos (IMO) foi quase zero na linhagem resistente, e na cv. FM 966 sucetível: FR = 13, IG = 5, IMO = 5. Os J2 de *Meloidogyne incognita* penetraram igualmente em ambos os genótipos. No estudo histopatológico observou-se forte fluorescência azul ao redor do nematoide (reação de hipersensibilidade, RH), principalmente no início (de 2-6 DAI) no córtex e no cilindro central das plantas resistentes, indicando acúmulo de compostos fenólicos nas raízes. Aos 9 DAI, células gigantes em fase inicial de subdivisão foram observadas próximas aos nematoides no cilindro central da planta resistente, mostrando também a presença de compostos fenólicos envolvendo o nematoide. Aos 12-40 DAI

estas células iniciais foram completamente degradadas com a presença de fenóis envolvendo os nematoides e células gigantes iniciais. Não foram observadas células gigantes completamente desenvolvidas nem fêmeas adultas. Apenas juvenis do quarto estágio (J4s) e machos foram visualizados aos 34 DAI. Este mecanismo de resistência caracteriza quase imunidade, sem fêmeas e sem produção de ovos. No controle suscetível foi possível visualizar sitios de alimentação bem desenvolvidos de 6 a 30 DAI. As fêmeas atingiram a maturidade aos 26 DAI e a produção de ovos aos 30 DAI. Nossos resultados sugerem que a resistência (próxima à imunidade) da linhagem CNPA17-26 B2RF foi relacionada a respostas de defesa precoces (2-12 DAI) que impediram totalmente a reprodução do nematoide.

Palavras-chave: Resistência de plantas; resposta hipersensível; nematoide das galhas.

1. INTRODUCTION

The root-knot nematode (RKN) *Meloidogyne incognita* (Kofoid & White, 1919), Chitwood, 1949, is a serious pathogen of cotton, causing direct damages and increasing the severity of other root diseases, in particular wilt disease caused by *Fusarium oxysporum* f.sp. *vasinfectum* (Jeffers & Roberts, 1993). 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 (Shepherd, 1983; Robinson and Percival, 1997; Robinson *et al*., 2004). Despite these efforts, few accessions with high level of resistance have been identified. In earlier studies, none of the accessions reached the level of resistance of

Auburn 623 RNR, Auburn 634 RNR and the later genotype M-315 that carry the resistant genes from Auburn 623. In addition to slower development of RKN in M-315, resistance is expressed by significantly fewer developing third and fourth stage juveniles at 8 DAI and fewer mature females at about 24 DAI. Reproduction on M-315 was so low that nematode population would be expected to decrease significantly under continuous cultures. M-315 possesses two major genes for resistance and is a valuable source of root-knot nematode resistance (Gutiérrez *et al*., 2010) incorporated to line CNPA17-26B2RF studied in this work.

Histological observations of two resistant accessions (*G. barbadense* CIR 1348 and *G. hirsutum* TX 25) showed that resistance occurs through a two-stage mechanism, in the first accession and through single stage mechanism in the second. Parasitism is blocked early after second–stage juvenile (J2) penetration or during its initial tissue migration (CIR 1348) and the development of later-stage juveniles into female adults is suppressed at a later stage (CIR 1348 and *G. hirsutum* TX 25). Fluorescence and bright field microscopy showed that root cells surrounding nematodes exhibited a hypersensitivity-like reaction (HR), with the accumulation of phenolic compounds and the presence of necrotic cells that limited the development of nematodes and the development of giant cells. Underdeveloped giant cells displaying a degenerated cytoplasmic content were found in small numbers in CIR 1348 and in a large number in TX-25, along with deformed nematodes (Mota *et al*., 2012).

In this study (Chap. 3), we present results on the screening of cotton genetic resources to identify novel sources of resistance to the root-knot nematode. This screening allowed the identification of cotton lines with high levels of resistance, as CNPA17-26 B2RF, equaling that of the most resistant lines known to date (M-315 RNR, a derivative of Auburn 623 RNR). The aim of this study was to characterize at

the histological level the near immunity resistance of genotype CNPA2017-26 B2RF of *G. hirsutum* to *M. incognita.* The penetration and post-infective development of this nematode in this resistant genotype was compared with the susceptible genotype FM966 to clarify the nematode biological cycle in both, and the resistance mechanism of CNPA17-26 B2RF line.

2. MATERIALS AND METHODS

2.1 Plant material

The genotypes used in the study were obtained from the Work Collection of Embrapa Cotton Breeding Program from EMBRAPA COTTON RESEARCH CENTER. The line CNPA17-26 B2RF was derived from the triple crossing of (BRS 368 RF x M-315) x BRS 430 B2RF], where BRS 368 RF and BRS 430 B2RF are sources of resistance to cotton blue disease (CBD) and to bacterial blight (BB) and M-315 to *M. incognita*. Seeds of the genotypes were placed in 500 ml plastic pots containing sterile sand and maintained under greenhouse conditions (25-30 ºC). The seedlings were supplied with a controlled-release complex fertilizer (West Garden, Raiz) and irrigated with this fertilizer (NPK+micronutrients) as needed.

2.2 Nematode population

The population of *M. incognita* used in this study was collected from cotton fields in Barreiras, Bahia State, Brazil (population 8 studied in chapter 2). Species identity was confirmed by using the esterase phenotype (Carneiro & Almeida, 2001) and SCAR markers. The nematode was multiplied in susceptible tomato plants cv Santa Clara during four months. The eggs were extracted from infected roots using a 0.5% NaOCl solution according to the protocol of Hussey & Barker (1973). However, a

blender for thirty seconds instead of manual agitation was used in this study to release eggs from the egg masses. The eggs were placed in a Baermann's funnel to hatch. The required amount of second-stage juveniles (J2) inoculum was obtained by daily collecting freshly hatched J2 and storing at 4 °C for a maximum of 4 days before using in the histopathological experiments. Ten thousand J2 were inoculated per seedling by pipetting nematode suspension around the stem base.

2.3 Histopathology

Seedlings of resistant and susceptible genotypes were grown and inoculated as described above. Roots samples were collected at 2, 4, 6, 9, 12, 15, 19, 23, 26, 30, 34 and 40 days after the inoculation (DAI) and washed with tap water. 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 (Axiophot Zeiss). Other parts of the 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. Sample dehydration was carried out under agitation in an increasing ethanolic series of 10-100%, with intervals of 20 min between the solution 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 thick. Unstained roots 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. Section images were recorded with a digital camera (AxioCam MRc – Zeiss).

3. RESULTS

3.1 Histopathology of the compatible interaction

Microscopical 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 apical meristem at 2 DAI (Figure 7 A). At 4 DAI some J2 reached the root central cylinder (CC), when it was possible to observe asymmetric plant cells, presumably due to cellular disorder after interaction with the nematode (Figure 7 B). Cell disorder was visualized at 2, 4, 6 and 8 DAI. At 6 DAI, oval-shaped, hypertrophied giant cells were observed (Figure 7C) adjacent to well-developed enlarged juveniles. The J3 changed into J4 at approximately 12-19 DAI (Figure 7D). At 19 DAI, J4 were found in well-established feeding sites (Figure 7E). Well-developed adult females were visualized at 30 DAI close to giant cells with thickened walls and some nuclei (Figure 7 F), 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-infection occurred as well.

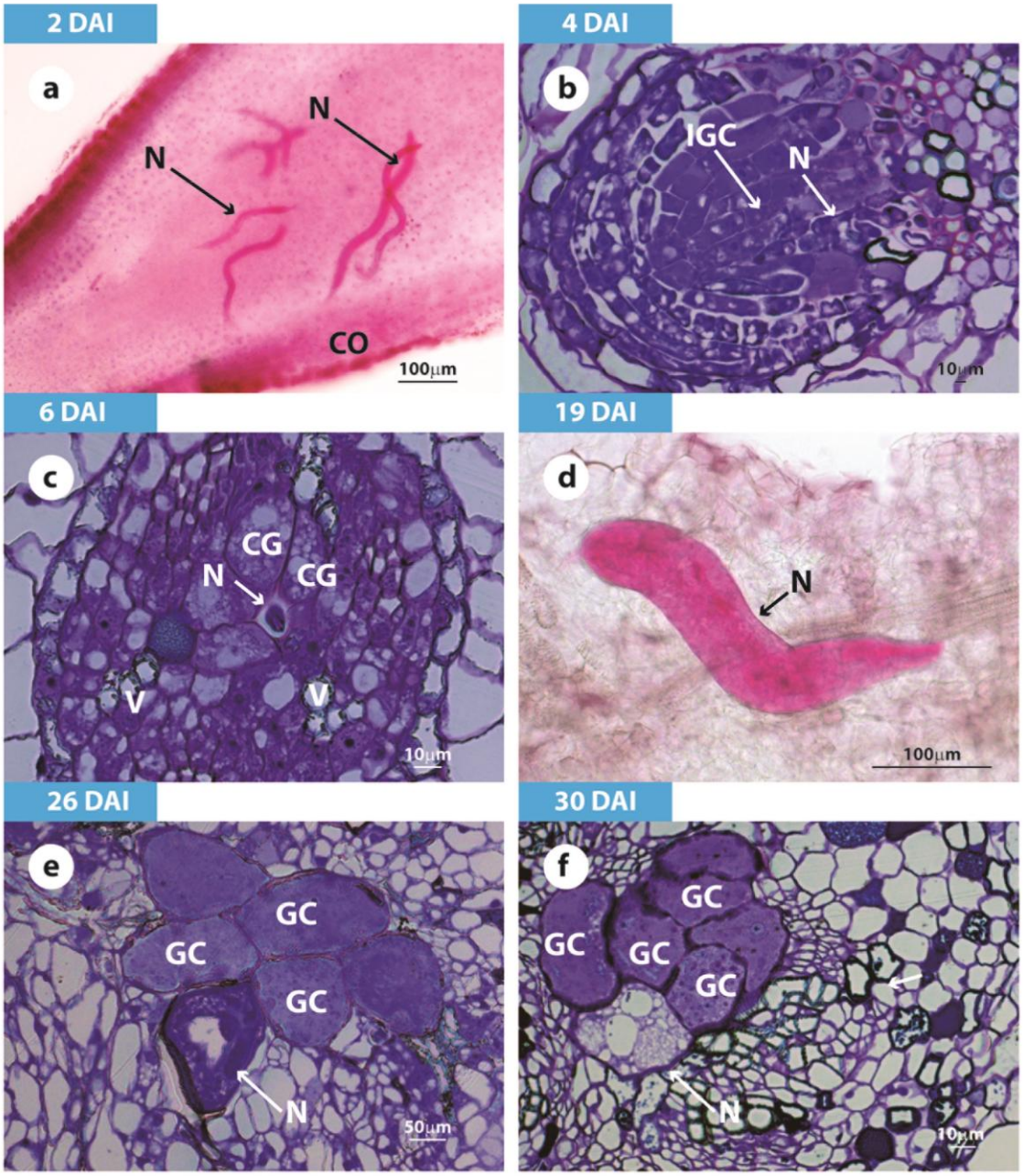

Figure 7. Roots of *Gossypium hirsutum* cv FM 966 (susceptible control) infected with *Meloidogyne incognita*. A and D stained with acid fuchsin (af); B, C, E, and F stained with toluidine blue. A – nematode (J2) migrating towards the central cylinder at 2 days after inoculation (DAI); B – initial giant 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.

3.2 Histopathology of the incompatible interaction

Observations made using the Byrd *et al*. (1983) methodology showed that 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. Only after this migratory phase at 6 DAI, J2s had already penetrated the central cylinder, and the nematode became sedentary. From 12-30 DAI, J3s and J4s were visualized in central cylinder. Some J4 females (Figure 8 F) and numerous males (Figure 8 G) were observed at 34 DAI, indicating that a high number of juveniles developed into males and not in pear shaped females. Mature root galls containing adult females and egg masses were not observed at 40 DAI.

In the resistant accession CNPA17-26 B2RF fluorescence microscopy using UV excitation of root sections harvested at 6 DAI showed a strong blue autofluorescence (UV) in several infection sites examined (Figure 8A). Sections visualized under bright fields microscope after toluidine staining showed numerous cells in the initial phase of division in dark blue staining, indicating necrosis and cell death at 9-12 DAI (Figure 8B, D). A strong blue fluorescence was observed, indicating accumulation of phenolic compounds, which is an indicative of hypersensitive reaction (HR) in roots in early stages (9-12 DAI) of infection (Figure 8C, E). At 12 DAI a total degeneration of cells during initial cell division was observed (Figure 8D) with accumulation of phenolic compounds (Figure 8E). At 23-40 DAI the root tissues adjacent to nematode feeding site was completely degraded with the presence of cell death and HR: dark blue (Figure 8H) and light or fluorescent blue (Figure 8I). There was also pink staining indicating unidentified polysaccharides (Figure 8H). No giant cell or egg mass was observed.

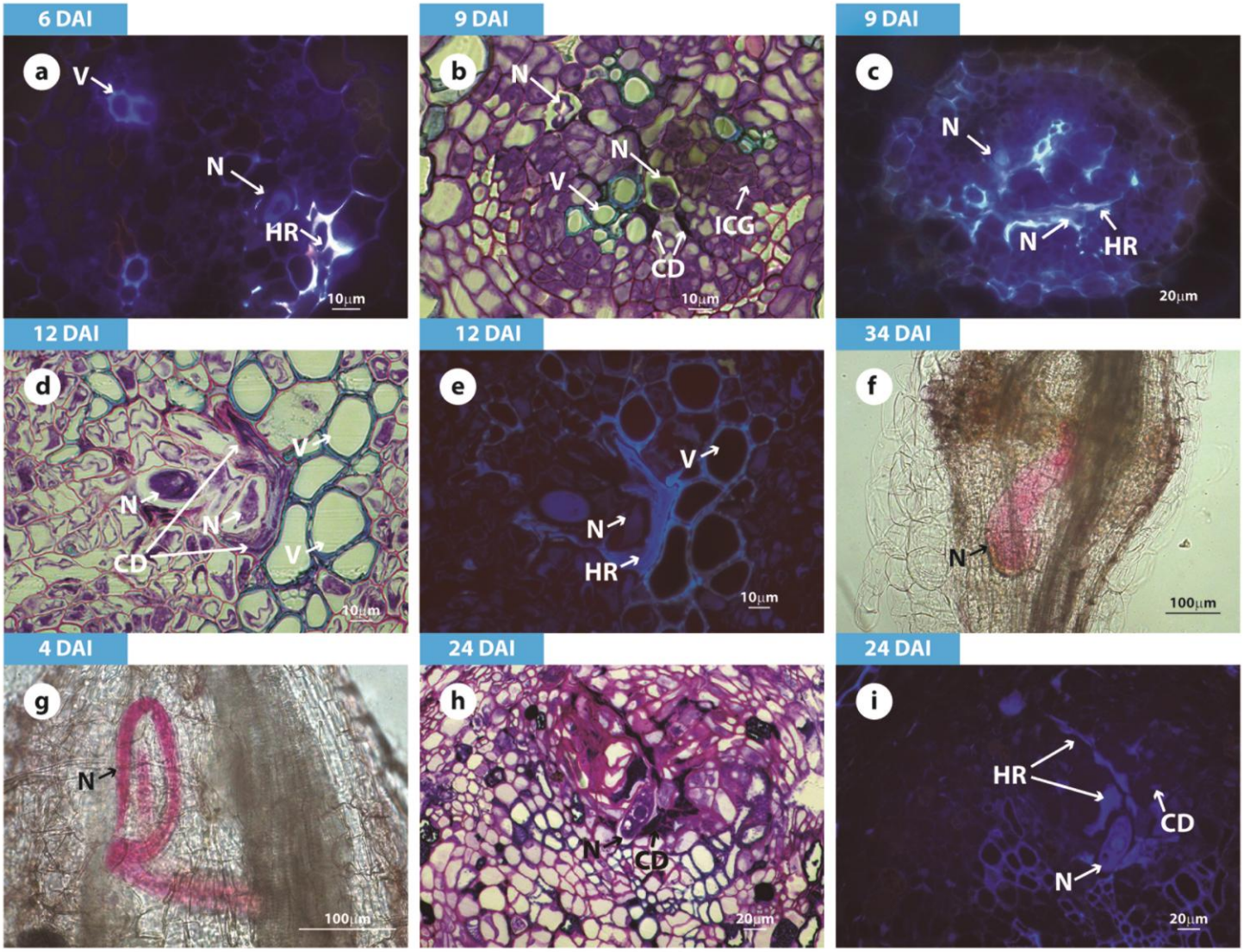

Figure 8. Roots of *Gossypium hirsutum* accession CNPA17-26B2RF (resistant) infected with *Meloidogyne incognita*. A, C, E – UV fluorescence observation. B, D, H – toluidine blue staining. F, G – acid fuchsin staining. A – a strong fluorescence (hypersensitivity reaction, HR) in the root central cylinder 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.

4. DISCUSSION

The resistance to *M. incognita* detected in *G. hirsutum* line CNPA17-26B2RF (studied in chapter 3) was investigated under greenhouse test and histopathological

observations. The reproduction factor (RF) of *M. incognita* in roots of the resistant accession was about zero and characterized as near immune (FR=0) compared with the susceptible FM 966. A strong incompatible plant response was observed, such as accumulation of phenolic compounds, cell death and failure to the nematode to develop into mature females and produce eggs 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 (Anwar & McKenry, 2002 Bendezu & 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 & Starr, 2003). While in the post-infection resistance, nematodes are able to penetrate the roots, but fail to develop (Anwar & McKenry, 2002). In this study, based on microscope observations, apparently the amount of J2 that penetrated the roots of susceptible and resistant accessions was similar, and penetration occurred in both. Jenkins *et al*. (1995) and Mota *et al*. (2013) observed the same in the genotype M-315 of *G. hirsuntum* and CIR1348 of *G. barbadense*, respectively. Pre-existing mechanisms which could prevent nematode penetration are apparently absent in cotton, in contrast with a number of situations in which reduced penetration in resistant plants were reported (Pegard *et al*., 2005; Proite *et al*., 2008).

In resistant accession CNPA 2017-26 B2RF, two different mechanisms could be involved in the expression of resistance. One occurred at 2 – 6 DAI, which blocks J2s after root penetration, as observed in others RKN–resistant cotton accession (Mota *et al*., 2013). 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, the *qMi-C11* affects the gall formation and *qMi-C14* is associated with reduced egg production (Silva *et al*., 2019; Gutiérrez *et al.*, 2010).

Histological analysis showed that in line CNPA17-26 B2RF, this early defense reaction was concomitant with observation of an HR–like response. The same mechanism was detected in CIR1348 (Mota *et al*., 2014). This response was shown to be involved in resistance to other RKN in a number of 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 presence of phenolic compounds, that could have a role in cotton defense to *M. incognita* (Nicholson & Hammerschmidt, 1992). Pegard *et al*. (2005) identified chlorogenic acid as the major phenolic compound present in root extract of inoculated RKN-resistant pepper and suggested this acid is prevent the nematode survival and its oxidation product significantly reduced their oxygen consumption.

The second later defense mechanism in CNPA17-26 B2RF line occurred at about 9-12 DAI and did not allow the formation of giant cells and females, therefore there was no egg production (equivalent to immunity). This second hypersensitive response (HR) occurred in the central cylinder involving nematodes and initial giant cells, and it was the most common mechanism as post-infection event associated with a rapid host cell death surrounding initial infection sites by the nematodes. As results, the pathogens is arrested and its development is completely inhibited (Williamson & Kumar, 2006) and visible signs of deterioration occurred, leading to initial giant cells collapsed at 12-40 DAI.

In general, immunity (FR=0) has been linked with early and late resistance mechanisms with initial HR and/or deterioration of the well-formed giant cells in resistant cotton (Mota *et al*., 2012) or resistant wild guava (Freitas *et al*., 2014).

Male sex conversion as it was frequently observed in this study, occurs when juveniles cannot establish appropriate feeding sites, and nutritional conditions are unfavorable to nematode development (Fassuliotis, 1970; Williamson & Hussey, 1996; Pofu & Mashela, 2011). In this study, presence of males can be explained by the fact of no formation of completely developed giant cells (GC), and, only initial ones providing suboptimal nutrition for female development at 26-30 DAI.

An epistatic interaction (M-315 and other resistant lines) between the two genes confers a near-immunity resistance to the RKN in the cotton genotypes carrying both genes, which could not be explained only by an additive effect of the two genes individually (Mcpherson *et al*., 2004; Shen *et al*., 2006, 2010; Ynturi *et al*., 2006; Gutiérrez *et al*., 2010; Jenkins *et al*., 2012; He *et al*., 2014).

Present results clarified that cotton accession line CNPA17-26 B2RF is an extremely efficient source of resistance, since it prevents the formation of giant cells and females, compromising totally reproduction of *M. incognita* race 3. Line CNPA17-26 B2RF has good agronomic traits, in addition harbors biotechnological events (Bollgard II: resistance against insects and Roundup Ready Flex – B2RF: tolerance to glyphosate), therefore, it can be launched as a cultivar and/or to serve as a source of genotypes adapted to tropical environment, as a parental donor of RKN resistance QTLs, and resistance for other economical important diseases in Brazil.

REFERENCES

Anwar SA, McKenry MV, 2002. Penetration and development of *Meloidogyne arenaria* on two new grape rootstocks. *Journal of Nematology* **34**, 143-145.

Bendezu IF, Starr JL, 2003. Mechanism of resistance to *Meloidogyne arenaria* in the peanut cultivar COAN. *Journal of Nematology* **35**, 115-118.

Byrd JDW, Kirkpatrick T, Barker KR, 1983. An improved technique for clearing and staining plant tissues for detection of nematodes. *Journal of Nematology* **15**,142-143.

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

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

Freitas VM, Correia VR, Motta FC, Sousa MG, Gomes ACMM, Carneiro MDG, Silva DB, Mattos JK, Nicole M, Carneiro RMDG, 2014. Resistant accessions of wild *Psidium* spp. to *Meloidogyne enterolobii* and histological characterization of resistance. *PlantPathology* **63**, 738–746.

Gutiérrez OA, Stelly DM, Saha S, Jenkins JN, Mccarty JC, Raska DA, Scheffler BE, 2010. SSR markers closely associated with genes for resistance to root- knot nematode on chromosomes 11 and 14 of Upland cotton. *Theoretical and Applied Genetics* **121**, 1323-1337.

Hussey RS, Barker KR, 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. *Plant Disease Reporter* **57**, 1025-1028.

Jeffers DP, Roberts PA, 1993. Effect of planting date and host genotype on the root-knot nematode-*Fusarium* wilt disease complex of cotton. *Phytopathology* **83**, 645–654.

Jenkins JN, Mccarty JC, Wubben MJ, Hayes R, Gutierrezo A, Callaha F, Deng D, 2012. SSR markers for marker assisted selection of root-knot nematode (*Meloidogyne incognita*) resistant plants in cotton (*Gossypium hirsutum* L). *Euphytica* **183**, 49-54.

Jenkins JN, Creech RG, Tang B, Lawrence GW, McCarty JC, 1995. Cotton resistance to root-knot nematode: II. Post-penetration development. *Crop Science* **35**, 369-373.

Lima EA, Furlanetto C, Nicole M, Gomes ACMM, Almeida MRA, Jorge-Junior A, Correa VR, Salgado SML, Ferrão MA, Carneiro RMDG, 2015. The multi-resistant reaction of drought-tolerant coffee Conilon clone 14 to *Meloidogyne* spp. and late hypersensitive-like response in *Coffea canephora*. *Phytopathology* **105**, 805-814.

He Y, Kumar P, Shen X, Davis RF, Van Becelaere G, May OL, Nichols RL, Chee PW, 2014. Re-evaluation of the inheritance for root-knot nematode resistance in the Upland cotton germplasm line M-120 RNR revealed two epistatic QTLs conferring resistance. *Theoretical and Applied Genetics* **127**, 1343-1351.

McPherson MG, Jenkins JN, Watson CE, Jr MC, 2004. Inheritance of root-knot nematode resistance inM-315 RNR and M-78 RNR cotton. *Journal of Cotton Science*, **8**, 154–161.

Mota FC, Alves GCS, Giband M, Gomes ACMM, Sousa FR, Mattos VS, Barbosa VHS, Barroso PAV, Nicole M, Peixoto JR, Rocha MR, Carneiro, RMDG, 2013. New sources of resistance to *Meloidogyne incognita* race 3 in wild cotton accessions and histological characterization of the defense mechanisms. *Plant Pathology* **62**, 1173-1183.

Nicholson RL, Hammerschmidt R, 1992. Phenolic compounds and their role in disease resistance. *Annual Review of Phytopathology* **30**, 369-389.

Pegard A, Brizzard G, Fazari A, Soucaze O, Abad P, Djian-Caporalino C, 2005. Histological characterization of resistance to different root-knot nematode species related to phenolics accumulation in *Capsicum annuum*. *Phytopathology* **95**, 158–165.

Proite K, Carneiro R, Falcão R, Gomes A, Leal-Bertioli S, Guimarães P, Bertioli D, 2008. Post-infection development and histopathology of *Meloidogyne arenaria* race 1 on *Arachis* spp. *Plant Pathology* **57**, 974-980.

Pofu KM, Mashela PW, 2011. Using relative penetration and maleness indices in *Meloidogyne incognita* to establish resistance type in *Cucumis myriocarpus*. *African Journal of Biotechnology* **10**, 390-393.

Robinson AF, Percival AE, 1997. Resistance to *Meloidogyne incognita* race 3 and *Rotylenchulus reniformis* in wild accessions of *Gossypium hirsutum* and *G. barbadense* from Mexico. *Journal of Nematology* **29**, 746-55.

Robinson AF, Bridges AC, Percival E, 2004. New source of resistance to the reniform (*Rotylenchus reniformis* Linford and Oliveira) and root-knot (*Meloidogyne incognita* Kofoid &White, Chitwood) nematode in Upland (*Gossypium hirsutum* L.) and Sea Island (*G. barbadense* L.) cotton. *The Journal of Cotton Science* **8**, 191– 197.

Shen X, He Y, Lubbers EL, Davis RF, Nichols RL, Chee PW, 2010. Fine mapping QMi-C11 a major QTL controlling root-knot nematodes resistance in Upland cotton. *Theoretical and Applied Genetics* **121**, 1623-1631.

Shen X, Van Becelaere G, Kumar P, Davis RF, Chee P, 2006. QTL mapping for resistance to root-knot nematodes in the M-120 RNR Upland cotton line (*Gossypium hirsutum* L.) of the Auburn 623 RNR source. *Theoretical and Applied Genetics* **113**, 1539-1549.

Shepherd RL, 1983. New sources of resistance to root-knot nematodes among primitive cottons. *Crop Science* **23**, 999-1002.

Silva MBDa, Davis RF, Kumar P, Nichols RL, Chee PW, 2019. Resistance QTLs qMi-C11 and qMi-C14 in cotton have different effects on the development of *Meloidogyne incognita*, the southern root-knot nematode. *Plant Disease* (first look).

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

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

Ynturi P, Jenkins JN, MccartyJC, Gutierrez OA, Saha S, 2006. Association of root-knot nematode resistance genes with simple sequence repeat markers on two chromosomes in cotton. *Crop Science* **46**, 2670-2674.

CONCLUSÕES E CONSIDERAÇÕES FINAIS

Com a execução do presente trabalho foi possível concluir que:

Populações do nematoide *Meloidogyne incognita* geneticamente divergentes ocorrem naturalmente no campo e essa diversidade pode estar, em alguns casos, relacionada com um aumento de agressividade à planta hospedeira. Não foram observadas, populações virulentas frente aos genótipos resistentes. A detecção de populações altamente agressivas na região produtora do Oeste da Bahia alerta para a necessidade de adoção de medidas de controle que previnam o surgimento de populações virulentas nas áreas produtoras de algodão, destacando a importância da rotação de culturas.

Os marcadores moleculares SSR associados aos QTLs das fontes de resistência M-315 e CIR 1348 testados no presente estudo, mostraram-se altamente eficientes para seleção de linhagens de algodão resistentes ao ataque do nematoide *M. incognita,* no entanto, foi identificada a necessidade de um mapeamento fino da segunda fonte, para que não ocorram falhas no uso da estratégia de seleção assistida por marcadores – MAS.

O estudo histopatológico da linhagem CNPA17-26B2RF revelou um poderoso mecanismo de resistência, caracterizado por forte reação de hipersensibilidade tanto em estágios iniciais do parasitismo do nematoide quanto em fases mais avançadas, tornando as plantas praticamente imunes ao nematoide, sendo este tipo de mecanismo nunca antes reportado no algodoeiro.

O conhecimento gerado pela execução deste trabalho contribui com o programa de melhoramento genético da EMBRAPA Algodão na busca de cultivares altamente resistentes a *M. incognita,* originadas de fontes variadas de resistência. Também destaca a importância da evolução do uso de marcadores moleculares no auxilio da seleção de

genes de resistência, como a utilização de marcadores que oferecem maior cobertura do genoma, como os SNPs, sendo os próximos passos a serem seguidos na pesquisa.