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Occurrence of *Meloidogyne* spp. in Cerrado Vegetations and Reaction of Native Plants to *Meloidogyne javanica*

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Abstract

The Cerrado biome represents a hotspot of biodiversity. Despite this, the nematofauna in this biome has not been well characterized, especially that related to root-knot nematodes. This work aimed to identify *Meloidogyne* species present in different cerrado vegetations and to investigate potential hosts of *Meloidogyne javanica* in this biome. Soil samples (250) were collected in native areas of cerrado vegetation located at the National Park of Brasília (PNB) (125 samples) and Água Limpa Farm (FAL) (125 samples), and transferred to sterile pots. Single tomato plants cv. Santa Clara (susceptible) were transplanted into individual pots and maintained for 90 days under glasshouse. Females of *Meloidogyne* spp. were extracted from tomato roots and identified based upon esterase phenotypes and confirmed with PCR using specific sequence characterized amplified regions (SCAR) primers. Native plants were inoculated with 10 000 individuals (eggs + J2) of a pure culture of *M. javanica* and maintained under glasshouse for 6 months. From the 250 samples collected, 57 (22.8%) presented *Meloidogyne* spp. A total of 66 *Meloidogyne* populations were identified as follows: *M. javanica* (75.76%), *M. incognita* (10.60%), *M. hapla* (9.1%), *M. morocciensis* (3.03%) and *M. arenaria* (1.51%). The following esterase phenotypes were detected: *M. javanica* (J3 and J2), *M. incognita* (I1 and I2), *M. hapla* (H1), *M. morocciensis* (A3) and *M. arenaria* (A2). The SCAR primers incK14F/incK14R, Fjav/Rjav and Fh/Rh amplified specific fragments in *M. incognita* (399 bp), *M. javanica* (670 bp) and *M. hapla* (610 bp) and can be used for identification of indigenous *Meloidogyne* spp. from cerrado. The primer set Far/Rar is not specific for *M. arenaria* due to the amplification of DNA in *M. morocciensis*. *Mimosa caesalpiniifolia* was the only native plant in which *M. javanica* developed a high reproductive rate, and it is probably a host for this nematode in cerrado.

Introduction

Cerrado is the second largest and richest Brazilian biome in terms of fauna and flora biodiversity, after the Amazon rainforest, and is composed of diverse types of vegetation, which together constitute the Cerrado sensu lato (Eiten 1972). In native Cerrado, certain vegetations dominate in relation to others such as 'seasonal forest' (cerradão), cerrado *sensu stricto*, 'closed field' (campo cerrado), 'dirty field' (campo sujo), 'clean field' (campo limpo) and 'gallery forest' (floresta de galeria) (Oliveira-Filho and Ratter 2002).

Besides its great diversity, the nematofauna present in native areas of cerrado vegetation has been poorly characterized, particularly in relation to root-knot nematodes (RKN) that are obligate endoparasites, causing losses to major crops worldwide. These plant-parasitic nematodes are considered the most

important and most abundant in agriculture, given their wide host-ranges, world distribution and interaction with other plant pathogens causing complex diseases (Moens et al. 2009).

In the last four decades, cerrado lands have become important production areas for major crops and pastures, which resulted in high rates of deforestation leading to fragmentation in natural resources and disturbances in the ecosystem (Goulart et al. 2003). Proper characterization of biodiversity in these natural biomes is strategically important for sustainable development and land use. Additionally, the documentation of RKN species and hosts associated with them present in areas of cerrado may contribute to choose better control management strategies for these new cropping areas within this biome.

The goals of this work were to identify *Meloidogyne* species present in soil samples covered by cerrado vegetations based upon esterase phenotyping, validate the applicability of sequence characterized amplified regions (SCAR) markers designed for different *Meloidogyne* spp. and investigate the reaction of native cerrado plants to *Meloidogyne javanica*.

Material and methods

Occurrence of *Meloidogyne* spp. in native cerrado

Sampling

Samples were taken from January 2010 to May 2011 in protected areas of native Cerrado at the Brasília National Park (PNB) and Ecological Station of Água Limpa Farm (FAL), the ecological reserve of the University of Brasília. Both places are located near the city of Brasília, Federal District, Brazil.

The sites composed of square plots of 50 × 50 m (2.500 m^2) were randomly established in selected areas characterized by cerrado vegetations. Such types of vegetation included Cerrado *sensu stricto*, closed field, clean field, dirty field and gallery forest. The establishment of square plots at each type of vegetation was repeated five times in different areas.

Five collection points (subsamples) were arranged within each plot, one located at the centre and four at the corners. Subsamples containing 300 grams of soil were collected at 0–20 cm depth and combined to form a composite sample of 1.5 kg that was used to grow tomato plants as a strategy to seek for *Meloidogyne* spp. A total of five composite samples (25 subsamples) were collected from each type of vegetation at PNB and FAL. For all the collection points, cartographic coordinates were determined by a GPS Garmim Mapping Map 60csx with a positional accuracy ≤10 m.

Reproducing native populations of Meloidogyne spp. on tomato plants

The soil collected from each plot was individually used into 1.5 kg plastic pots. Individual tomato plants (cv. Santa Cruz KaDa) at the five-leaf stage were transplanted into each pot under glasshouse. Ninety days after transplanting, tomato roots were removed from the soil, washed and examined for the presence of galls. Egg masses and single females were used for biochemical and molecular diagnostics. When in mixtures, *Meloidogyne* populations were purified through the inoculation of single egg masses on tomato plants. Forty-one *Meloidogyne* populations that reproduced on tomato plants had their DNA extracted for PCR analysis.

Identification of Meloidogyne spp. by esterase phenotype

Single young females were extracted from tomato roots and identified by esterase phenotype. Electrophoresis was conducted in 7% polyacrylamide gels run in a horizontal CL18 Permatron gel tank. Isoenzymes were electrophoresed for 2 h at 4°C and 80 volts, according to Carneiro and Almeida (2001).

Diagnostic PCR

Sequence characterized amplified regions markers developed by Zijlstra (2000), Zijlstra et al. (2000) and Randig et al. (2002) were used for *Meloidogyne* spp. detection (Table 1). Eggs of *Meloidogyne* species were extracted from tomato roots 90 days after inoculation, with total DNA extracted from 300 μl of eggs (Randig et al. 2002).

Reaction of native plants to *M. javanica*

Inoculum preparation

A population of *M. javanica* was selected based upon its aggressiveness on tomato plants, prior to inoculations on native plants. *Meloidogyne javanica* was multiplied on tomato plants for 3 months as described previously. For egg extraction, tomato roots were cut into small pieces (1 cm) and blended for 1 min in a 0.5% sodium hypochlorite (NaOCl), according to the technique adapted from Hussey and Barker (1973). Eggs were counted in a light microscope using Peters' slide and the final volume adjusted to a concentration of 10 000 eggs per ml.

Inoculation of native plants with M. javanica

The treatments (native plants and tomato) were cultivated individually in 5 kg polyethylene bags filled with substrate (soil + sand, 1 : 1 v/v) and fertilized monthly with 2 g of NPK (4-14-8). Plants with 20–30 cm height were inoculated with 10 000

Table 1 Sequence characterized amplified regions markers tested against indigenous populations of *Meloidogyne* spp. from the Brazilian cerrado

Primer SCAR	Sequence (5′→ 3′)	Amplicons (bp)	Reference	Target species
inc-K14-F	GGGATGTGTAAATGCTCCTG	399	Randig et al. (2002)	*M. incognita*
inc-K14-R	CCCGCTACACCCTCAACTTC			
Far	TCGGCGATAGAGGTAAATGAC	420	Zijlstra et al. (2000)	*M. arenaria*
Rar	TCGGCGATAGACACTACAACT			
Fjav	GGTGCGCGATTGAACTGAGC	670	Zijlstra et al. (2000)	*M. javanica*
Rjav	CAGGCCCTTCAGTGGAACTATAC			
Fh	TGACGGCGGTGAGTGCGA	610	Zijlstra (2000)	*M. hapla*
Rh	TGACGGCGGTACCTCATAG			

M. javanica eggs and maintained under greenhouse for 6 months with temperatures ranging from ~ 20 to 35°C. The inoculum was equally distributed into four holes around the stem.

Experimental design and evaluation of inoculated plants

The experimental design was complete randomization of the 16 treatments with five replications. The treatments included native plants of cerrado as *Amburana cearensis* (Freire All.) Smith, *Dalbergia miscolobium* Benth., *Dimorphandra mollis* Benth., *Enterolobium gummiferum* (Mart.) Macb., *Eugenia dysenterica* Mart. ex DC., *Hymenaea stigonocarpa* Mart. Ex Hayne, *Kielmeyera coriacea* Mart. ex Saddi, *Lafoensia pacari* St. Hil., *Magonia pubescens* St. Hil., *Mimosa caesalpiniifolia* Benth., *Ormosia arborea* (Vell.) Harms., *Qualea grandiflora* Mart., *Solanum lycocarpum* St. Hil., *Stryphnodendron adstringens* mart. coville and *Handroanthus impetiginosus* (Mart. Ex DC.) Mattos, besides the positive control *Solanum lycopersicum* L. (tomato). Six months after inoculation, the treatments were evaluated based upon the following parameters: gall index, egg masses index, eggs per gram of roots and reproduction factor (RF). Gall index and egg masses index were analysed according to this scale (Taylor and Sasser 1978): 0 = complete absence; 1 = 1–2; 2 = 3–10; 3 = 11–30; 4 = 31–100; 5 = above 100. These numbers correspond to both gall and egg masses.

Results

Survey of *Meloidogyne* spp. in cerrado vegetation

From 250 soil samples collected in both preserved areas, PNB and FAL, 57 (22.8%) of those samples were infested with *Meloidogyne* spp. The occurrence and frequency of *Meloidogyne* species within these areas were variable among species. *Meloidogyne javanica* was the most abundant species observed (75.76%), followed by *M. incognita* (10.60%), *M. hapla* (9.1%), *M. morocciensis* (3.03%) and *M. arenaria* (1.51%) (Table 2). Most *Meloidogyne* species in both cerrado areas were found in single populations (91.5%), with few samples showing mixtures of *Meloidogyne* species (8.5%). Moreover, 45% of the *Meloidogyne* populations were found in clean field, followed by gallery forest (22.5%), closed field (22.5%) and Cerrado *sensu stricto* (10%). *M. javanica* was detected in all cerrado vegetations, *M. morocciensis* in Cerrado *sensu stricto* and clean field, while *M. incognita* was detected in dirty field, clean field and gallery forest, and *M. hapla* and *M. arenaria* in dirty field (Table 2).

Esterase phenotypes

Considering the different esterase phenotypes found in *M. javanica* and *M. incognita* populations, their distribution was variable among the cerrado vegetations studied in this work. *Meloidogyne javanica* EST J3 was found in all cerrado vegetations, while *M. javanica* EST J2 phenotype was detected only in gallery forest and closed field (Table 2, Fig. 1 a,b,c). *M. incognita* EST I1 was detected in gallery forest while *M. incognita* EST I2 was found in gallery forest, dirty field and clean field (Table 2, Fig. 1 e,f). Other esterase phenotypes observed in this study comprised EST A3 for *M. morocciensis* , EST A2 for M. arenaria and EST H1 for *M. hapla* (Table 2, Fig. 1 g,h)

Identification by SCAR markers

Species-specific SCAR primers (Table 1), used individually or in multiplex reactions, were validated for indigenous populations of *M. incognita* (399 bp), *M. morocciensis* (420 bp), *M. javanica* (670 bp) and *M. hapla* (610 bp) (Fig. 2), previously identified based upon esterase phenotype. The resolution of SCAR-PCR was insufficient for detection of intraspecific variability in populations of *M. incognita* (EST I1 and EST I2) and *M. javanica* (EST J2 and EST J3).

Table 2 Detection of *Meloidogyne* spp. by esterase phenotypes and validation of SCAR markers for indigenous populations of *Meloidogyne* from cerrado

Site[a]	Vegetation type[b]	Enzyme phenotypes[c]			SCAR marker[d]
		Esterase (EST)	Number of populations	*Meloidogyne* species	*Meloidogyne* species
PNB	Cerrado *sensu stricto*	A3	1	*M. morocciensis*[e]	–
PNB	Clean field	A3	1	*M. morocciensis*	*M. morocciensis*
PNB	Closed field	J3	1	*M. javanica*[e]	–
PNB	Closed field	J3	1	*M. javanica*	*M. javanica*
PNB	Clean field	J3	3	*M. javanica*	*M. javanica*
PNB	Gallery forest	J3	2	*M. javanica*	*M. javanica*
FAL	Cerrado *sensu stricto*	J3	3	*M. javanica*	*M. javanica*
FAL	Cerrado *sensu stricto*	J3	1	*M. javanica*[e]	–
FAL	Closed field	J3	1	*M. javanica*	*M. javanica*
FAL	Closed field	J3	2	*M. javanica*[e]	–
FAL	Closed field	J2	1	*M. javanica*[e]	–
FAL	Closed field	J3	14	*M. javanica*	*M. javanica*
FAL	Clean field	J3	2	*M. javanica*[e]	–
FAL	Gallery forest	J3	1	*M. javanica*[e]	–
FAL	Gallery forest	J3	1	*M. javanica/M. incognita*	*M. javanica/M. incognita*
FAL	Gallery forest	J2	1	*M. javanica*	*M. javanica*
FAL	Clean field	J3	1	*M. javanica/M. incognita*	*M. javanica/M. incognita*
FAL	Dirty field	J3	2	*M. javanica*	*M. javanica*
FAL	Dirty field	J3	5	*M. javanica*[e]	–
FAL	Clean field	J3	1	*M. javanica/M. hapla*	*M. javanica/M. hapla*
FAL	Clean field	J3/I2	2	*M. javanica/M. incognita*[e]	*M. javanica*
FAL	Clean field	J3/I2	1	*M. javanica/M. incognita*	*M. javanica/M. incognita*
FAL	Dirty field	J3/A2/H1	1	*M. javanica/M. arenaria/M. hapla*[e]	*M. javanica*
FAL	Dirty field	J3/H1	2	*M. javanica/M. hapla*[e]	*M. javanica*
FAL	Dirty field	J3/H1	1	*M. javanica/M. hapla*[e]	–
FAL	Gallery forest	I2	1	*M. incognita*	*M. incognita*
FAL	Clean field	I2	1	*M. incognita*[e]	–
FAL	Gallery forest	I1	1	*M. incognita/M. javanica*	*M. incognita/M. javanica*
FAL	Dirty field	I2	1	*M. incognita*[e]	–
FAL	Dirty field	H1	1	*M. hapla*	*M. hapla*
FAL	Dirty field	H1	1	*M. hapla/M. javanica*	*M. hapla/M. javanica*
Total number of populations			58		
Number/(%) of populations per species					
M. javanica			50 (75.76%)		
M. incognita			7 (10.60%)		
M. hapla			6 (9.10%)		
M. morocciensis			2 (3.03%)		
M. arenaria			1 (1.51%)		

[a]PNB and FAL represent two conserved areas of Cerrado near Brasília, Central Brazil.
[b]Designates different types of cerrado vegetation.
[c]Esterase phenotype labelling after Esbenshade and Triantaphyllou (1985).
[d]Molecular characterization of *Meloidogyne* spp. using species-specific primers (SCAR-PCR), according to references listed in Table 1.
[e]Indigenous populations of *Meloidogyne* with poor development on tomato roots that were not tested with the scar markers.

Reaction of native plants to *M. javanica*

From 15 native plants inoculated with *M. javanica*, only *Mimosa caesalpiniifolia* showed a high reproductive rate (RF = 22.73) of this nematode, with all other plant species with RFs between 0 and 0.25. The control plants (tomato) showed the highest RF (125.43) (Table 3), as expected. This is the first report of *M. caesalpiniifolia* as a host for *M. javanica*.

Discussion

Meloidogyne javanica was present in 75.76% of the total samples collected in native cerrado, including

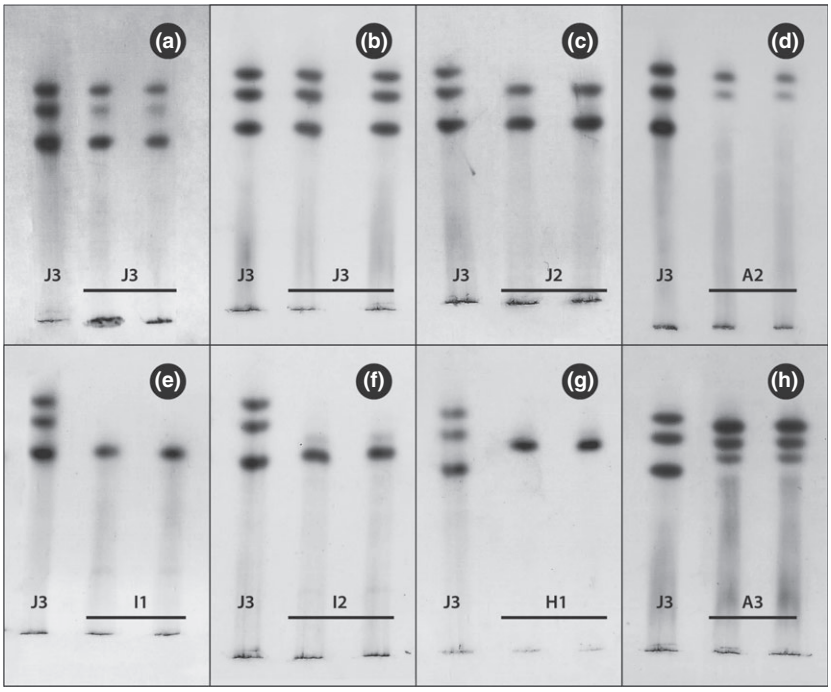

Fig. 1 Esterase phenotypes of *Meloloidogyne* spp. from areas of Cerrado in the Central Plateau of Brazil. (a) *M. javanica* (J3) with the second band slightly faint; (b) *M. javanica* (J3); (c) *M. javanica* (J2); (d) *M. arenaria* (A2); (e) *M. incognita* (I1); (f) *M. incognita* (I2); (g) *M. hapla* (H1) and (h) *M. morocciensis* (A3). *M. javanica* (J3) was used as a standard esterase phenotype.

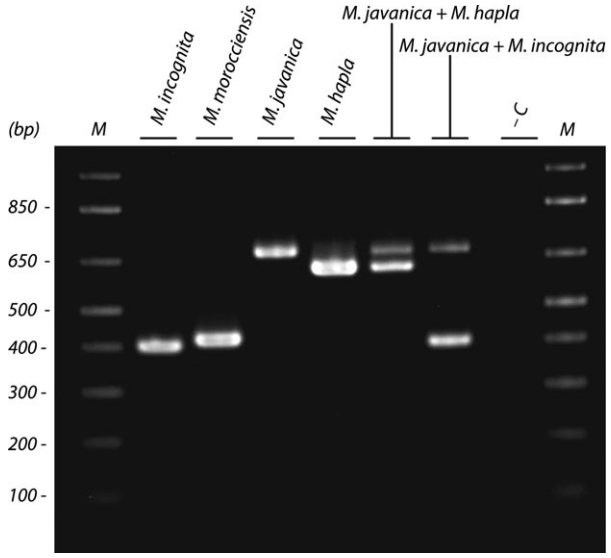

Fig. 2 Amplification pattern of *Meloidogyne* spp. by sequence characterized amplified regions (SCAR-PCR). *Meloidogyne incognita* (399 bp), *M. morocciensis* (420), *M. javanica* (670), *M. hapla* (610) and mixed populations of *M. javanica* + *M. hapla* and *M. javanica* + *M. incognita*; bp: base pairs, M: 1 kb DNA plus ladder, -C: negative control, minus template DNA.

the detection of two esterase phenotypes, EST J2 and EST J3. Previous survey for RKN nematodes in native cerrado reported *M. javanica* EST J3 as the most prevalent species (85%) (Souza et al. 1994). In the Atlantic Forest biome, *M. javanica* was reported as the main species detected (Lima et al. 2005), while in Western Paraná, Brazil, *M. javanica* was also reported parasitizing roots of native plants from the Atlantic Forest (Antes et al. 2012). The frequency of *M. javanica* in native soils covered by cerrado vegetation in Brazil can be explained by its wide host-range and broad distribution (Hunt and Handoo 2009).

The presence of variants EST J2 and EST J3 in populations of *M. javanica*, the latter with a clearer intermediate band has already been observed by Carneiro et al. (1996). The detection of *M. hapla* in the cerrado of the Brazilian Federal District extends the list of *Meloidogyne* species associated with this biome, as there has been no previous report of this nematode in the cerrado. *Meloidogyne hapla*, the northern RKN, typically occurs in temperate climates and at higher altitudes in the tropics (Hunt and Handoo 2009). In Brazil, it has been reported in cooler southern regions (Carneiro et al. 1996, 2000) and its detection in the Brazilian Federal District may reflect the altitudes of this region (ranging from 600 to 1300 m).

Meloidogyne arenaria and *M. morocciensis* (=*M. arenaria* EST A3) are closely related species and had been detected previously in areas of cerrado by Souza et al. (1994). Later, *M. arenaria* was reported causing galls on the roots of a native cerrado plant (Carneiro et al. 2006), but the origin of the nematode population was unknown. The primer set Far/Rar tested in this work was confirmed to be

Table 3 Reaction of plants from native cerrado vegetation inoculated with 10 000 eggs of *Meloidogyne javanica* and assessed 6 months after inoculation

Plant species	Family	Fresh root mass[a]	GI[b]	EMI[c]	Total number of eggs	Total number of eggs/gram of root	RF[d]
Amburana cearensis (Freire All.) Smith	Fabaceae	33	0	0	0	0	0
Dalbergia miscolobium Benth.	Fabaceae	7. 1	0	0	0	0	0
Dimorphandra mollis Benth.	Caesalpiniaceae	5. 17	0	0	0	0	0
Enterolobium gummiferum (Mart.) Macb.	Fabaceae	19. 9	1	0. 8	86. 66	4. 24	0
Eugenia dysenterica Mart. ex DC.	Myrtaceae	3. 6	0	0	0	0	0
Hymenaea stigonocarpa Mart. Ex Hayne	Fabaceae	18. 7	0	0	0	0	0
Kielmeyera coriacea Mart. ex Saddi	Clusiaceae	6. 5	0	0	0	0	0
Lafoensia pacari St. Hil.	Lythraceae	14. 5	0	0	0	0	0
Magonia pubescens St. Hil.	Sapindaceae	12. 83	0	0	0	0	0
Mimosa caesalpiniifolia Benth.	Mimosaceae	36. 33	4. 66	4. 66	227. 333. 33	6331. 79	22. 73
Ormosia arborea (Vell.) Harms.	Fabaceae	7. 0	2	1. 4	2286. 67	620. 90	0. 22
Qualea grandiflora Mart.	Vochysiaceae	5. 25	0	0	0	0	0
Solanum lycocarpum St. Hil.	Solanaceae	96. 1	1	1	2320	24. 20	0. 23
Stryphnodendron adstringens mart. coville	Fabaceae	2. 33	0	0	0	0	0
Handroanthus impetiginosus (Mart. Ex DC.) Mattos	Bignoniaceae	14. 4	1. 2	0. 8	386. 66	175. 22	0
Solanum lycopersicum L. (tomato)	Solanaceae	56. 5	5	5		23 061. 28	125. 43

[a]Average of fresh root mass (g).
[b]Gall index (Taylor and Sasser 1978).
[c]Egg mass index (Taylor and Sasser 1978). 0 = no gall or no egg mass; 1 = 1–2 gall(s) or 1–2 egg mass(es); 2 = 3–10 galls or 3–10 egg masses; 3 = 11–30 galls or 3–10 egg masses; 4 = 31–100 galls or 31–100 egg masses; 5 = above 100 galls or egg masses.
[d]RF, reproduction factor (final nematode population/initial nematode population), calculated based on an average of 5 replicates per treatment. .

non-species-specific for *M. arenaria* as suggested by Carneiro et al. (2008).

Despite being one of the most widespread and polyphagous species of *Meloidogyne*, *M. incognita* had its first report for cerrado in this work. *Meloidogyne incognita* had been reported before in the Atlantic Forest biome in Rio de Janeiro, Brazil (Lima et al. 2005) and in Western Paraná, Brazil (Antes et al. 2012).

The SCAR markers used in this work did not resolve *Meloidogyne* populations with distinct isozyme phenotypes, but were efficient in the detection of indigenous populations of *Meloidogyne* spp. The efficiency of these markers was previously reported by Zijlstra (2000), Zijlstra et al. (2000) and Randig et al. (2002).

Five species of *Meloidogyne* were found in the native cerrado of the Federal District region, two of them being new reports for the Cerrado biome (*M. incognita* and *M. hapla*). Out of these species, *M. javanica* was by far the most widespread, followed by *M. incognita*. The collection points at the experimental areas were demarcated by GPS to monitor these nematode populations in further studies.

The presence of *Meloidogyne* spp. in the Brazilian cerrado indicates that these nematodes can feed and reproduce on the roots of cerrado vegetation. *Mimosa caesalpiniifolia* is a plant found in natural biomes in Brazil such as Caatinga (semi-arid areas in Brazil) and Cerrado. In cerrado areas of Piauí state, *M. caesalpiniifolia* is commonly found in vegetation composed of savanna, Cerrado *lato sensu* and woodland. Moreover, this plant species shows potential for use in animal nutrition, honey production (due to the high production of nectar and pollen for attracting bees), as a source of energy (wood coal) and stake, etc. (Carvalho 2007).

The high reproductive rate of *M. javanica*, detected in the roots of *M. caesalpiniifolia*, indicates that this plant is extremely sensitive to this nematode and could contribute to the survival of *M. javanica* in areas of cerrado. The presence of *Meloidogyne* species in native cerrado will be reflected in the health of crops when cerrado areas are replaced by agriculture (Lehmann et al. 1977). This indicates the importance of conducting surveys in cerrado agrosystems aiming to discover whether RKN are present, prior to recommending appropriate crops to be cultivated or other control methods to manage *Meloidogyne* spp.

Protection of native cerrado will contribute to maintenance of biodiversity in this ecosystem, whose *Meloidogyne* species are components. Inoculation of

native cerrado plants with indigenous populations of *Meloidogyne* is an alternative approach to identification of hosts of this nematode in the Cerrado biome, as the detection of natural infection in collecting sites remains a challenge.

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