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Responses of Guava Plants to Inoculation with Arbuscular Mycorrhizal Fungi in Soil Infested with *Meloidogyne enterolobii*

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In the Northeast of Brazil, expansion of guava crops has been impaired by *Meloidogyne enterolobii* that causes root galls, leaf fall and plant death. Considering the fact that arbuscular mycorrhizal Fungi (AMF) improve plant growth giving protection against damages by plant pathogens, this work was carried out to select AMF efficient to increase production of guava seedlings and their tolerance to *M. enterolobii*. Seedlings of guava were inoculated with 200 spores of *Gigaspora albida*, *Glomus etunicatum* or *Acaulospora longula* and 55 days later with 4,000 eggs of *M. enterolobii*. The interactions between the AMF and *M. enterolobii* were assessed by measuring leaf number, aerial dry biomass, CO₂ evolution and arbuscular and total mycorrhizal colonization. In general, plant growth was improved by the treatments with *A. longula* or with *G. albida*. The presence of the nematode decreased arbuscular colonization and increased general enzymatic activity. Higher dehydrogenase activity occurred with the *A. longula* treatment and CO₂ evolution was higher in the control with the nematode. More spores and higher production of glomalin-related soil proteins were observed in the treatment with *G. albida*. The numbers of galls, egg masses and eggs were reduced in the presence of *A. longula*. Inoculation with this fungus benefitted plant growth and decreased nematode reproduction.

Keywords : enzymatic activity, glomeromycota, *Psidium guajava*, root knot

In the lower middle region of the São Francisco Valley, located in the States of Pernambuco and Bahia, Northeast Brazil, characterized by semi-arid climate with mean temper-

ature of 27°C and 350–800 mm annual precipitation, the irrigated areas with fruit crops have shown promising development especially concerning guava (*Psidium guajava* L.). However, the parasitism of the nematode *Meloidogyne enterolobii* a sedentary endoparasite which causes root galls, browning of the leaf edge and yellowing of the aerial shoots leads to general defoliation and plant death (Carneiro et al., 2001). This plant pathogen causes high losses and plantation abandonment. One of the promising alternatives for root-knot control could be the use of arbuscular mycorrhizal fungi (AMF) which in association with the plant roots improves nutrient uptake and increase plant development, protecting the host against biotic and abiotic stresses (Maia et al., 2006). Meta analysis performed by Borowicz (2001) based on studies published between 1970 and early 1998 has shown that AMF decrease nematode performance, indicating increased resistance, with this harmful effect being extensive only to sedentary nematodes.

The positive effect of AMF on growth of guava plantlets has been demonstrated (Schiavo and Martins, 2002), as well as interactions of nematode and AMF in different crops. Such interactions depend upon the plant species, the AMF isolate, the nematode involved and the cultivation conditions (Diedhiou et al., 2003; Elsen et al., 2003). In the particular case of *Meloidogyne* species, the plants associated with AMF can induce decrease in number of root galls and eggs of the nematode, and, as a consequence, growth improvement and development are observed leading to reduction in damages with increase in production (Jaizme-Vega et al., 1997).

Considering that there are no reports regarding a tripartite interaction including AMF, nematodes and guava plants, the hypothesis that the inoculation with these fungi can reduce the damage caused by *M. enterolobii* was tested. Therefore, the aim of this work was to select AMF efficient in increasing production of guava plantlets and tolerance to

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the guava root-knot nematode.

Materials and Methods

Soil classified as loamy sand (collected in Petrolina municipality, State of Pernambuco, in the Caatinga biome, close to the Embrapa Semi-arid Experimental Station) was treated with Bromex® (98% methyl bromide and 2% chloropicrin) and allowed to rest for 15 days before use. The soil, analyzed at the Embrapa Soil Laboratory, has the following chemical characteristics: pH 6.9; 23 mg dm⁻³ of P; 0.69 cmol_c dm⁻³ of K; 3.9 cmol_c dm⁻³ of Ca; 0.9 cmol_c dm⁻³ of Mg; 0.19 cmol_c dm⁻³ of Na; 12.0 g dm⁻³ of organic matter and 6.34 cmol_c dm⁻³ of cation exchange capacity.

Guava (*P. guajava* cv. Paluma) plantlets (90 days old rooted cuttings) were acquired from the Brazil Plantlets Company in Petrolina-PE. In the laboratory, the plantlets were removed from the substrate (a mixture of sawdust, vermiculite and guava commercial substrate), and the roots washed with water and transferred to black polyethylene bags with 2 kg of the aforementioned soil. The inoculum was composed by 200 spores, hyphae and colonized roots of the following AMF: *Gigaspora albida* N. C. Schenck & G. S. Sm. (UFPE 01), *Glomus etunicatum* W. N. Becker & Gerd. (UFPE 06) or *Acaulospora longula* Spain & N. C. Schenck (UFPE 21) produced in a sand + vermiculite substrate and multiplied in association with *Panicum miliaceum* L., was applied to the rhizosphere of each plant. These plantlets were maintained under greenhouse conditions (average temperature: 27 ± 2 °C; relative humidity: 75%; luminosity: 250 to 560 μmole/m²/s) and 55 days after inoculation with the AMF, received a suspension of 4,000 eggs and juveniles of *M. enterolobii*, extracted following the Hussey and Barker (1973) method. The nematode was obtained from infected guava roots, collected at the Tamba Farm (BR 428, Km 154, Petrolina, PE).

The experiment was conducted using a completely randomized design in factorial scheme with: 4 AMF treatments (inoculated with *A. longula*, *G. albida* or *G. etunicatum* and an uninoculated control) × 2 nematode treatments (presence or absence of *M. enterolobii*) in 5 replicates. The experimental results were examined 98 days after inoculation with AMF, and the following variables were evaluated: plant height, number of leaves, stem diameter, dry and fresh biomass of the aerial part, fresh root biomass, leaf area, mycorrhizal colonization, AMF spore density, production of glomalin-related soil proteins (GRSP), numbers of galls, egg masses and eggs, total and per gram of root, CO₂ evolution, dehydrogenase activity and global soil enzymatic activity.

Dry biomass was obtained after oven drying (60 °C) until constant weight. Leaf area was measured with a Li 3100

device (LI-Cor Inc. Lincoln, Neb., USA). Roots were cleared in 10% KOH, stained with 0.03% Chlorazol Black (Brundrett et al., 1984) and the percentage of hyphae, arbuscules and vesicles were evaluated (McGonigle et al., 1990). Spores of AMF were extracted from the soil by wet sieving and centrifugal flotation (Gerdemann and Nicolson, 1963; Jenkins, 1964) and quantified with a stereomicroscope (40 ×). Glomalin-related soil proteins were extracted as described by Wright and Upadhyaya (1996) and quantified by the Bradford (1976) method. Nematode eggs were extracted using 1 cm root fragments immerse in 1% sodium hypochlorite for 4 minutes in constant shaking (Hussey and Barker, 1973). The counting was made using a light microscope.

Dehydrogenase activity was evaluated by the Casida et al. (1964) method, the CO₂ content determined according to Grisi (1978) and general soil enzymatic activity analyzed by fluorescein diacetate hydrolysis (Swisher and Carroll, 1982).

Data were submitted to analysis of variance and the values in percentage (mycorrhizal colonization) and in numbers (AMF spore density, number of galls, egg masses and eggs) were transformed into arcsin x/100 and in log x+1, respectively. For determining statistical significance, mean values were compared by the Tukey test at 5% probability. The analyses were carried out using Statistica 6.0 software (Statsoft, 1997).

Results

In plants without the nematode, the number of leaves did not differ between the control (without AMF) and plants inoculated with *G. albida* and *A. longula*. However, in the presence of the pathogen, the number of leaves was higher in the treatment with *A. longula* (Table 1). For dry biomass of the aerial part, differences occurred only in plants inoculated with *G. etunicatum* which were smaller than those of the other treatments when the pathogen was present (Table 1).

Fresh root biomass was significantly higher in AMF inoculated plants; whereas larger leaf area was observed in plantlets inoculated with *A. longula* and *G. albida*. However, only those associated with *A. longula* presented greater stem diameter than the control (Table 2).

Higher activity of soil dehydrogenase occurred in the treatment with *A. longula* (Table 2). Global enzymatic activity was higher in the presence of the nematode (0.90 and 0.86 μg of fluorescein g⁻¹ dry soil, respectively for treatments with and without nematodes) with the same occurring for CO₂ evolution, except for the treatment with *A. longula* (Table 1).

In the absence of the nematode higher rates of respiration

Table 1. Plantlet growth data, rhizosphere microbial activity and mycorrhizal colonization in roots of guava in the treatments: uninoculated (control) and inoculated with AMF, in the absence (–) or presence (+) of *Meloidogyne enterolobii*, 98 days after inoculation with AMF, under greenhouse conditions

Treatment	Leaf number		Dry biomass of aerial parts (g)		CO ₂ (µg C-CO ₂ /g dry soil/day)		Arbuscule colonization (%)		Total colonization (%)	
	–	+	–	+	–	+	–	+	–	+
Control	23 aA	17 bB	6.59 aA	7.53 aA	2.21 abB	9.26 aA	10 dA	14 cA	42 bA	23 bA
<i>Acaulospora longula</i>	20 abB	28 aA	8.97 aA	6.99 aA	4.04 aA	2.27 cA	35 bA	26 bB	62 aA	40 abB
<i>Gigaspora albida</i>	26 aA	18 bB	7.49 aA	5.74 abA	1.57 bB	5.87 bA	46 aA	38 aB	48 abA	42 aA
<i>Glomus etunicatum</i>	16 bA	16 bA	7.70 aA	4.05 bB	0.50 bB	4.87 bA	27 cA	18 bcB	32 bA	38 abA

Mean values followed by the same lower case letters, in the column, and capital letters in the row, do not differ according to the Tukey test at 5% probability

in the soil were detected in the treatment with *A. longula*. For the treatments with nematode, higher rates of respiration were observed in the control (Table 1).

In spite of the attempt to sterilize the soil, the non inoculated control was colonized by AMF. In the treatment without nematodes, *A. longula* produced more colonization than the control, whereas in the presence of the pathogen, this occurred in the treatment with *G. albida*. The presence of *M. enterolobii* decreased the colonization produced by *A. longula* in the inoculated plants (Table 1).

Arbuscules were the colonization structures more affected by the presence of the nematode. In the absence of *M. enterolobii*, all treatments differed statistically, with inoculated roots presenting more arbuscular colonization than the

control. Among the inoculated treatments, that with *G. albida* had more colonized roots, even in the presence of *M. enterolobii* (Table 1). Regardless of the presence of the nematode and compared to the control, colonization by hyphae was greater in roots inoculated with *G. albida*, whereas more vesicles were formed in roots associated with *A. longula* (Table 2).

The nematode did not affect glomerospores production and GRSP (glomalin-related soil proteins) (Table 2). *G. albida* produced more glomerospores than the other AMF and higher glomalin content was observed in the rhizosphere of plants inoculated with this fungus, when compared with the *G. etunicatum* treatment (Table 2).

The total number of galls, egg masses and eggs did not

Table 2. Plantlet growth variables, dehydrogenase activity, glomalin content, AMF spore density in the rhizosphere and colonization by vesicles and by hyphae in guava roots in the treatments uninoculated (control) and inoculated with AMF, regardless of the inoculation with *Meloidogyne enterolobii*, 98 days after inoculation with AMF in the greenhouse

Treatments	Diameter (cm)	Fresh root Biomass (g)	Leaf area (cm ²)	Dehydrogenase (µg TTF /g dry soil)	Glomalin (mg glomalin /g soil)	Spore density (50/g soil)	Colonization by vesicles (%)	Colonization by hyphae (%)
Control	0.438 b	5.17 b	445.27 b	0.043 b	1.034 ab	0.3 d	0.14 b	3.2 b
<i>Acaulospora longula</i>	0.585 a	10.46 a	708.77 a	0.062 a	1.024 ab	1.8 c	0.92 a	3.4 b
<i>Gigaspora albida</i>	0.521 ab	11.75 a	677.94 a	0.033 b	1.185 a	46.8 a	0.00 b	49.0 a
<i>Glomus etunicatum</i>	0.508 ab	9.21 a	497.25 b	0.037 b	0.929 b	10.8 b	0.26 ab	11.0 b

Mean values followed by the same letter in the column do not differ according to the Tukey test at 5% probability

Table 3. Variables related to the nematode (*Meloidogyne enterolobii*) in roots of guava plants, with or without AMF, 98 days after inoculation with the fungi, and maintained in a greenhouse

Treatments	Number of galls (total)	Number of galls (/g root)	Number of egg masses (total)	Number of egg masses (/g root)	Number of eggs (total)	Number of eggs (/g root)
Control	207.2 a	32.4 a	169.0 a	30.2 a	44.524 a	7.093 a
<i>Acaulospora longula</i>	181.4 a	15.0 b	85.4 a	11.6 b	17.671 a	1.658 b
<i>Gigaspora albida</i>	175.2 a	28.8 a	128.6 a	19.2 ab	38.478 a	5.362 a
<i>Glomus etunicatum</i>	285.8 a	46.0 a	88.0 a	20.6 ab	6.455 a	1.246 b

Mean values followed by the same letter in the column do not differ according to the Tukey test at 5% probability.

significantly differ but when the quantities per gram of root were considered, the treatments with *A. longula* had lower amounts, and that with *G. etunicatum* also presented a lower number of eggs, compared to the control (Table 3).

Discussion

Significant interactions were found between AMF and *M. enterolobii* in number of leaves, dry biomass of aerial plantlet parts. This was also true for total mycorrhizal colonization, arbuscules and CO₂ evolution in the soil. The leaf area differed significantly in the nematode treatments or with AMF, but there was no interaction. Stem diameter and fresh root biomass, soil dehydrogenase activity, GRSP, AMF spore density and mycorrhizal colonization by hyphae and vesicles were affected only by the AMF treatments, whereas for the global enzymatic activity of the soil, a significant effect was only observed for the nematode.

Inside the roots, the root-knot nematodes (*Meloidogyne* spp.) create a feeding site deriving continuous nourishment from adjacent cells and producing galls that affect the plant's metabolism and resource allocation, impairing the absorption and transport of water and nutrients which results in decreased plant development (Borowicz, 2001; Carneiro et al., 2002). The presence of AMF, which compete for space and nutrients with the nematode, may reduce this effect, inducing plant development even in the presence of the pathogen, as observed in olive plants (*Olea europaea* L.) (Castillo et al., 2006), alpinia [*Alpinia purpurata* (Viell.) Schum] (Silva, 2005), papaya (*Carica papaya* L.) (Jaizme-Vega et al., 2006), sweet passion fruit (*Passiflora alata* Curtis) (Anjos et al., 2010) and other crops.

Different AMF can induce various responses in *Meloidogyne* infected plants, and the response of a plant species varies considerably with the identity of the symbiotic fungus (Smith et al., 2009). In tomatoes (*Lycopersicon esculentum* L.) infected with *Meloidogyne javanica* (Treub) Chitwood associated with *Gigaspora margarita* W. N. Becker & I. R. Hall the biomass of the aerial part, weight and number of fruits were similar to the control, while in presence of *Glomus etunicatum* these parameters were increased (Cofcewicz et al., 2001).

Guava plants associated with *G. albida* and *A. longula* presented greater growth than the other treatments. Despite the fact that there is no specificity between AMF and their hosts, greater compatibility can occur between some species of AMF and plants, and differences can be attributed to the genotype and the functional compatibility of the partners (Costa et al., 2001). Thus, AMF selection is very important for the establishment of an effective symbiosis. The literature presents an example showing that growth of sweet passion-fruit plantlets was more improved by inoculation

with *G. albida* than with *G. etunicatum* or *A. longula* (Silva et al., 2004). Also, inoculation with *G. margarita* and *Glomus intraradices* N. C. Schenck & G. S. Sm. enhanced biomass production of strawberry plantlets (*Fragaria ananassa* Duch.), whereas growth was impaired by other seven AMF species (Taylor and Harrier, 2001).

Higher activity of soil dehydrogenase in the treatment with *A. longula* may indicate higher production of hyphae by this fungus than by the other AMF, as detected by the dehydrogenase activity, considering that it reflects the oxidative potential of the soil (Gianfreda et al., 2005).

Nematode respiration may have contributed to greater metabolic activity and these organisms, as well as the roots they damage, are sources of organic matter, favoring respiratory and enzymatic activity of soil organisms, as reported by Fernandes et al. (2005). Plant parasite nematodes increase organic carbon availability in the soil and consequently microbial activity and CO₂ release (Tu et al., 2003); this fact occurs when the nematode perforates the root cell, leaving a hole through which carbon is drained and can be easily used by the microbial community. Plant nematodes may also facilitate the decomposition of organic matter due to partial degradation of cellulose considering that their enzymes break down this compound (Tu et al., 2003).

In the absence of the nematode higher rates of respiration in the soil were detected in the treatment with *A. longula*, confirming the results related with dehydrogenase activity and reinforcing the possibility of the presence of more hyphae of this fungus. For the treatments with nematode, higher rates of respiration were registered in the control, which could be related to the reduction of the nematodes population induced by the AMF and consequently, less respiration. Among the fungi, *Acaulospora longula* and *G. etunicatum* were more promising than *G. albida* in inducing a decrease in the population of nematodes in the roots, as shown by the number of galls, egg masses and eggs.

The intensity of mycorrhizal colonization can be affected by the presence of nematodes and varies according to the AMF species. In the rhizosphere of *Cucumis sativus* L. cv. Zhongnong 16 the presence of *Meloidogyne incognita* (Kofoid & White) Chitwood decreased colonization by *Glomus mosseae* (T. H. Nicolson & Gerd.) Gerd. & Trappe whereas colonization by *G. intraradices* was not affected by the nematode (Zhang et al., 2008). In white clover (*Trifolium repens* L.), *M. incognita* induced an increase in colonization by *G. intraradices*, without any effect in the colonization produced by *G. aggregatum* N. C. Schenck & G. S. Sm. and *G. mosseae* (Habte et al., 1999). From a meta-analysis including 90 experiments, Borowicz (2001) observed that presence of nematodes reduced AMF colonization in only 16% of them.

A correlation between the amount of glomerospores and

glomalin has been reported (Bedini et al., 2007), since this glycoprotein is one of the constituents of the spore wall, but this was not observed in this work.

The treatment with *G. etunicatum* presented a lower number of eggs, compared to the control. This can be related to the effects of the fungus on the development of the nematode, which penetration into the roots was high, inducing gall formation, but reproduction was low (number of eggs). A decrease in the body length of nematodes (*M. javanica*) and a smaller number of giant cells were reported in tomato plants infected by this pathogen and associated with *G. mosseae* (Siddiqui and Mahmood, 1998).

Considering that in plants associated with *A. longula* there was less penetration of the nematode and a lower reproduction rate (eggs per gram of root), the effects would be from the AMF pre-colonization, making the roots less attractive to the nematode. Therefore, some physiological alterations probably promoted by the AMF, such as modification of the chemical composition of root exudates and production of some compounds (phenylalanine, serine, phenols), may have an antagonistic effect toward the nematodes, as observed in other studies (Siddiqui and Mahmood, 1995). The reduction in penetration may also be due to changes in cell wall composition of the roots (Hol and Cook, 2005), as well as by the activation of plant defense mechanisms stimulated by the AMF (Azcón-Aguilar and Barea, 1996). Vos et al. (2011) observed less juveniles penetration in mycorrhizal roots of the *Solanum lycopersicum* L. cv. Marmande.

The effects of inoculation with AMF on the development and reproduction of nematodes depend on the fungus and plant species involved (Carling et al., 1996). In peanuts (*Arachis hypogaea*) the presence of *G. etunicatum* increased the number of galls in the roots and the production of eggs by *Meloidogyne arenaria* (Neal) Chitwood (Carling et al., 1996). The opposite occurred in *Musa* AAA cv. Grande Naine infected with *M. incognita* and inoculated with *G. mosseae* (Jaizme-Vega et al., 1997). However, in *Lycopersicon esculentum* L. cv. Tounvi the number of galls in the roots decreased, while the number of eggs increased in the treatments with *G. mosseae* or *Acaulospora spinosa* C. Walker & Trappe (Affokpon et al., 2011). The mechanisms involved in the AMF root knot nematode relationship and the effect of the host plant on both organisms are still not clear (Azcón-Aguilar and Barea, 1996); therefore, studies that elucidate this mode of action are very important in order to promote the biocontrol of these pathogens.

The effects of AMF on the growth of plants inoculated with *Meloidogyne* spp. are highly variable, but usually positive; the same occurring in regard to the establishment of the symbiosis and development of the nematode (Maia et al., 2006). Among the AMF treatments, that with *A. longula*

was the most promising because it not only induced a reduction in the amount of *M. enterolobii* eggs, but also was efficient in increasing plant growth. In the treatment with *G. etunicatum*, there was a decrease in the number of nematode eggs but no benefits for plant development and in plants associated with *G. albida*, no growth benefits or decrease in number of eggs were observed. In experiments with *A. purpurata* inoculation with the same isolate of *A. longula* used in this study, increased plant growth was observed, even in the presence of *M. arenaria*, with a decrease in the number of eggs (Silva, 2005), demonstrating the efficiency of this fungus in controlling root-knot nematodes. Thus, strategies for establishment of plantlets in the field and increase in the production of guava, even in the presence of nematodes, should include more studies with *A. longula* and other species, for selection of promising isolates that can be used in association with guava plants in semi-arid regions.

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