Interactions between arbuscular mycorrhizal fungi and *Meloidogyne incognita* in the ornamental plant *Impatiens balsamina*

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**Abstract**

Biocontrol traits of arbuscular mycorrhizal fungi (AMF), in terms of single and mixed species inoculum, against the root knot nematode *Meloidogyne incognita* in *Impatiens balsamina* L., were examined with and without mineral fertilization in a greenhouse pot experiment. At harvest, 60 days after sowing, general plant growth parameters and plant defense response in terms of antioxidant activity and content of phenolic compounds in roots and leaves were measured. Also AMF root colonization and abundance of nematode root-knots were determined. Mineral fertilization increased all plant growth parameters measured, which coincided with an increased disease development caused by *M. incognita*. Inoculation with AMF mitigated the observed plant growth reduction caused by *M. incognita*, though, higher abundance of *M. incognita* root knots was found in mycorrhizal plants. Plant defense responses in terms of antioxidant activity and content of phenolic compounds did not seem to be linked to the observed biocontrol traits of AMF against *M. incognita*. However, roots inoculated with a consortium of AMF, which presented less nematode root knots than roots with the single species inoculum, had the highest level of phenolic compounds. The results from the present study suggest that AMF induce tolerance in *I. balsamina* against the root knot nematode *M. incognita*.

**Keywords:** Root pathogen, biocontrol, plant defense, antioxidants, phenolic compounds

1. Introduction

Plant parasitic nematodes such as the root knot nematode *Meloidogyne incognita* represent a severe yield-limiting factor in plant production systems worldwide (Koenning *et al.* 2004). Measures to control disease include, sound crop rotation programs, development of resistant plant germplasm and application of nematicides (Chitwood 2002). However, nematicide application provokes undesired non-target effects, calling for plant disease management protocols based on biological resources (Winter 1999). AMF represents a well-known beneficial biological resource in relation to plant production in agroecosystems,
where improved host plant nutrition and health are among the most important ecosystem services provided (Gianinazzi et al. 2010). AMF have been shown to reduce development of root diseases caused by pathogens including oomycetes, fungi and nematodes (St. Arnaud and Vujanovic 2007; Veresoglou and Rillig 2012). Main proposed modes of biocontrol traits of AMF against root pathogens include competition for space and nutrients, antagonism from mycorrhiza associated bacteria and plant defense induction (St. Arnaud and Vujanovic 2007).

Interactions between AMF and plant parasitic nematodes depend on several factors including host plant, AMF and nematode species, but in general AMF may induce host tolerance and/or increase host resistance (Hol and Cook 2005). Indeed, AMF have been shown to reduce development of sedentary endoparasitic nematodes, whereas AMF may increase numbers of migratory endoparasitic nematodes (Hol and Cook 2005).

Plants subjected to pathogen stress activate metabolites and defense mechanisms (Holopainen and Gershenzon 2010). Several genes are activated and different compounds are produced as a response to these conditions, such as antioxidant and phenolic compounds (Xu et al. 2008). The antioxidants constitute a primary defense against free radicals generated during stress conditions (Wang et al. 2003), and they have been shown to protect roots against damage caused by root pathogens (Kangatharalingam et al. 2002). In addition, phenolic compounds may also activate reactive oxygen species neutralizers (Blokhina 2003).

In terms of plant defense it is well known that AMF root colonization can lead to increased levels of antioxidants and phenolic compounds Zhu and Yao 2004; Xu et al. 2008; Carlsen et al. 2008), though often transient and weak, compared to that of pathogens (Pozo and Azcón-Aguilar 2007).

The main objectives of this study were to examine interactions between AMF and the root knot nematode *M. incognita* in roots of fertilized and non-fertilized *I. balsamina* plants and to examine the effects of single and combined inoculations with AMF and nematodes on plant growth and defense response.

### 2. Materials and Methods

#### 2.1. Biological materials

Two types of AMF inocula were used both provided by the Laboratory of Beneficial Organisms, Universidad Veracruzana: single species inoculum with the AMF *Glomus coronatum* and the AMF species consortium “MTZUV” that containing eleven AMF species (*Acaulospora morrowiae*, *Acaulospora scrobiculata*, *Acaulospora spinosa*, *Claroideoglomus etunicatum*, *Funneliformis geosporus*, *Funneliformis mosseae*, *Gigaspora decipiens*, *Gigaspora rosea*, *Glomus aggregatum*, *Glomus macrocarpum* and *Scutellospora pellucida*). Both AMF inocula had been propagated with *Brachiaria decumbens* as host plant and consisted of soil, root segments, spores and mycelium. Prior to use the AMF inocula had been subjected to standard AMF inoculum quality tests. Ten grams of inoculum, containing a minimum of 300 spores per AMF species and other fungal structures inside the roots, were applied per pot by mixing it thoroughly in to the growth substrate. Inoculum of the root knot nematode *M. incognita* consisted of egg-masses collected from naturally *M. incognita* infected roots of wild *I. balsamina* in surrounding fields of Xalapa, Mexico. Approximately 500 juvenile nematodes (J2) were inoculated to 40 days old seedlings. Counting of the nematodes was performed with a Neubauer chamber.

#### 2.2. Experimental design

The experiment had a three-factorial design with “Fertilization” (2 levels: without and with), “Mycorrhiza” (3 levels: without, *G. coronatum* and
2.3. Experimental set-up and plant growth conditions

The experiment was set up as a greenhouse pot experiment (500 mL pot⁻¹) with an autoclaved sterilized soil: sand mix (2:1) as the growth substrate. The soil used was a commercial gardening soil from Gardens and Parks of Xalapa. The soil had a 6.04 pH, 22 mg kg⁻¹ of N, 4.4 mg kg⁻¹ of P, and 800 mg kg⁻¹ of Ca, and the organic matter content of the soil was 5.9%. The pots were filled with 400 g of substrate per pot. The experiment was conducted under greenhouse conditions with mean temperature of 32 °C (15-38 °C). Inocula of AMF were mixed into the growth media before sowing and nematodes were inoculated 40 days after sowing. Half of the plants received N-P-K fertilization (150-32-40 ppm) to each plant by means of nutrient solution and it was applied six times during the experiment, while the other half remained without fertilization. Plants were watered as needed.

2.4. Harvest and plant analyses

Plants were harvested 80 days after sowing and analyzed for plant growth parameters (shoot dry weight, root fresh weight, plant height and leaf area), plant defense measures (total antioxidant activity [TAOX], and the total content of phenolic compounds [TCPC] in leaves and roots) and microbial measures (number of nematode root-knots, and AMF root colonization).

The TAOX was performed using the DPPH (Sigma® D-9132) assay with Trolox (Sigma-Aldrich®) for preparing a calibration curve (Re et al. 1999; Matthäus 2002). Fresh leaf or root tissue (0.150 g) was ground in 3 mL of 80% methanol, and extracts were centrifuged at 15,000 rpm for 15 minutes. The reaction consisted of a mixture of 15 µL of the extract added with 250 µL of the DPPH solution in microplates with 96 wells. The absorbance readings were taken first at 515 nm, and then microplates were incubated for 15 minutes and a second reading was taken (Biotek Synergy 2 Spectrophotometer, Biotek®). The TAOX was expressed as μmol of Trolox g⁻¹ of fresh tissue.

TCPC were determined using the assay with the Folin-Ciocalteu reagent. Chlorogenic acid was utilized as standard for the calibration curve (Singleton and Rossi, 1965; Soong and Barlow 2004). The fresh leaf or root tissue (0.150 g) was ground in a mortar with 80% methanol. The extracts were centrifuged for 15 minutes at 15,000 rpm. The reaction mixture consisted on 30 μL of the plant extract adding 90 μL of Na₂CO₃ and 150 μL of Folin-Ciocalteau in microplates with 96 wells. After 30 minutes, the absorbance was measured at 725 nm (BiotekSynergy 2 Spectrophotometer, Biotek®). The TCPC was expressed as μg of chlorogenic g⁻¹ of fresh tissue.

AMF root colonization was determined by clearing and staining roots as described by Philips and Hayman (1970) and measured in accordance to the gridline procedure (Giovannetti and Mosse 1980). Root knot nematode number was counted using a stereo microscope.

2.5. Statistics analysis

Data were subjected to a three way analysis of variance after testing for variance homogeneity (Levene's test). Post ANOVA treatment means comparisons were based on LSD (α=0.05). All statistical analyses were performed with the software Statgraphics Centurion XV.

3. Results

3.1. Microbial variables

Significant two factor interactions, in terms of “Fertilization x Nematodes”, “Fertilization x Mycorrhiza” and “Nematodes x Mycorrhiza”, were observed for AMF root colonization (Table 1).

MTZUV) and “Nematodes” (2 levels: without and with) as the main factors. Each of the 12 treatments had three replicates providing a total of 36 experimental units.
Table 1. Levels of significance ($P$) of main factors and their interactions obtained from three way analyses of variance (n=3).

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ND indicates “not determined”; Fertilization (F), Mycorrhiza (M), Nematode (N); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, NS indicates “non-significant”.

Fertilization alone had no effects on the number of nematode root-knots (Figure 1a), but when fertilized, AMF inoculation increased the number of nematode root-knots (Figure 1a).

Regardless of nematode inoculation, roots presented high level of AMF colonization, between 50 and 80% (Figure 5b). In non-fertilized plants, inoculation with the two AMF inocula resulted in a similar AMF root colonization level (Figure 5b). Fertilization significantly increased the level of AMF colonization with the MTZUV inoculum when compared to that by $G. coronatum$ (Figure 1b). Nematode inoculation in non-fertilized plants resulted in increased root colonization with $G. coronatum$ (Figure 1b). Fertilization increased the AMF root colonization in plants inoculated with MTZUV. Nematode inoculation in fertilized plants reduced the AMF root colonization with $G. coronatum$, but had no effect on the AMF root colonization with MTZUV inoculum (Figure 1b).
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Figure 1. Total number of nematode root-knots (a) and AMF root colonization of fertilized (N-P-K) and non-fertilized I. balsamina as affected by single and combined inoculation with two types of AMF inoculum and the nematode M. incognita. Bars with different letters indicate significant differences between treatments (LSD≤0.05; n=3). I, standard error.

3.2. Plant growth variables

Each of the three main factors significantly affected all plant growth variables measured either independently or in terms of interactions between factors (Table 1).

A significant three way interaction (“Fertilization x Mycorrhiza x Nematodes”) was observed for shoot dry weight, which was overall increased by fertilization (Table 1, Figure 1a). Shoot dry weight of fertilized plants were unaffected by both AMF and nematode inoculation. In fertilized plants single inoculation with AMF and nematodes caused plant growth depressions, which were counteracted when AMF and nematode inoculation were combined (Figure 2a). Significant effects on root fresh weight were observed for all three main factors, but no interactions were observed between factors (Table 1). In non-fertilized plants inoculation with G. coronatum increased root fresh weight, whereas inoculation with the AMF consortium
MTZUV had no effect on root fresh weight (Figure 2b). Fertilization caused an increase in root fresh weight regardless of AMF inoculation compared to that of non-fertilized plants (Figure 2b). Nematode inoculation had no effect on root fresh weight in non-fertilized plants, whereas nematode inoculation in fertilized plants markedly reduced root fresh weight. Combined nematode and AMF inoculation in fertilized plants completely counteracted the reduction in root fresh weight observed with single nematode inoculation (Figure 2b).

Figure 2. Plant shoot dry weight (a) and root fresh weight (b) of fertilized (N-P-K) and non-fertilized *I. balsamina* as affected by single and combined inoculation with two types of AMF inoculum and the nematode *M. incognita*. Bars with different letters indicate significant differences between treatments (LSD≤0.05; n=3). I, standard error.
Significant interactions were observed for “Fertilization x Mycorrhiza” and “Mycorrhiza x Nematodes” in relation to leaf area (Table 1). Inoculation with AMF and nematodes had no effect on leaf area of non-fertilized plants. Leaf area of fertilized plants respectively increased and decreased with AMF and nematode inoculation. Combined inoculation with AMF and nematodes partially counteracted the reduction in leaf area caused by single inoculation with nematodes (Figure 3a). Significant effects of all three main factors “Fertilization”, “Mycorrhiza” and “Nematodes” were observed for plant height, whereas no significant interactions were observed between factors (Table 1). Overall, fertilization and AMF inoculation increased plant height, whereas nematode inoculation reduced plant height (Figure 3b).

Figure 3. Plant leaf area (a) and height (b) of fertilized (N-P-K) and non-fertilized I. balsamina as affected by single and combined inoculation with two types of AMF inoculum and the nematode M. incognita. Bars with different letters indicate significant differences between treatments (LSD≤0.05; n=3). I, standard error.
3.3. Plant defense variables

TAOX in shoot and root was unaffected by all three factors examined except that AMF inoculation significantly affected TAOX in shoots (Table 1, Figure 4a and 4b). Overall inoculation with the AMF inoculum MTZUV caused an increase in TAOX in shoot compared to that of non-mycorrhizal plants and to plants inoculated with *G. coronatum* (Figure 4a).

![Graph](image)

**Figure 4.** Total antioxidant content in shoot (a) and (b) roots of fertilized (N-P-K) and non-fertilized *I. balsamina* as affected by single and combined inoculation with two types of AMF inoculum and the nematode *M. incognita*. Bars with different letters indicate significant differences between treatments (LSD≤0.05; n=3). I, standard error.

Significant three way interaction (“Fertilization x Mycorrhiza x Nematodes”) was observed for TCPC in both shoot and root (Table 1). Fertilization had no effect on TCPC in shoot. Inoculation with nematodes increased TCPC, but this effect was mitigated by inoculation with AMF, where MTZUV inoculum further decreased TCPC, compared to that of plants without nematodes (Figure 4a).
In nematode inoculated plants, the TCPC in shoot increased and was counteracted by the MTZUV inoculum. On the other hand, nematode inoculation of fertilized plants decreased TCPC in shoot (Figure 5a).

In roots, TCPC was mainly affected by AMF inoculation (Figure 5b). Inoculation with *G. coronatum* in non-fertilized plants resulted in reduced TCPC when compared to treatments without AMF inoculation or with the MTZUV inoculum (Figure 5b). In fertilized plants inoculated with nematodes, the MTZUV inoculum caused a significant increase in TCPC in roots (Figure 5b).

4. Discussion

The obtained results indicate that AMF inoculation induced tolerance in fertilized *I. balsamina* against the pathogen *M. incognita*, which is in agreement with the general consensus that AMF can induce
tolerance against plant-parasitic nematodes (Hol and Cook 2005). However, the coinciding increase in *M. incognita* root knots deviates from the general idea that AMF reduce root infection level of sedentary endoparasitic nematodes due to competition for plant photosynthates (Hol and Cook 2005).

Mycorrhizal plants may be better hosts or be more attractive for pathogens than non-mycorrhizal plants, since they develop more biomass (Graham 2001), and may compensate plant growth suppression from biotrophic plant pathogens via increased photosynthetic activity (Gernns et al. 2001). Dual inoculation with AMF and the nematode *M. incognita* alleviated plant growth suppression caused by nematode infection in fertilized *I. balsamina* plants, supporting the compensation hypothesis presented by Gernns et al. (2001). In the present study mycorrhizal plants had a higher leaf area than non-mycorrhizal plants with a corresponding higher photosynthetic potential. In contrast, root knot nematode infection has been found to affect water uptake and photosynthetic activities, affecting plant growth and development (Siddiqui and Sayeed Akhtar 2009).

In general, AMF provide improved plant nutrition and health (Smith and Read, 2008), but in some cases host growth suppression may occur when net benefit from the symbiosis is higher than the cost in terms of plant photosynthates. In the present study both AMF inocula caused host growth suppressions in treatments with fertilization and without nematode inoculation, but provided clear biocontrol traits in terms of induced tolerance against root-knot nematodes. Similar biocontrol traits of plant growth suppressive AMF have been reported in tomato-*Glomus* symbioses (Larsen et al. 2012).

Potentially mycorrhizal *I. balsamina* may increase the soil inoculum potential of *M. incognita*, which is important to consider as a possible drawback from the observed AMF induced tolerance in *I. balsamina*. Similar considerations have been discussed by Thygesen et al. (2004) in relation to AMF induced tolerance of pea to root pathogens.

AMF have been shown to alter the abundance and composition of plant phenolic compounds (Carlsen et al. 2008). However, plant defense induction in terms of content of phenolic compounds and antioxidant activity does not seem to be strongly linked to the observed biocontrol trait of AMF obtained in the present study. Nevertheless, inoculation with AMF clearly affected plant defense reactions in terms of increased level of shoot antioxidants, and phenolic compounds in roots of fertilized plants when inoculated with the mixed species of MTZUV inoculum, where less nematode root knots were observed. Similar differential effects of AMF on plant defense compounds, in terms of root flavonoids, have been reported by Carlsen et al. (2009) in relation to AMF-Pythium interactions in white clover.

Recently a similar result was reported from Maya and Matsubara (2013), where they found an increased on the antioxidant production in AMF inoculated cyclamen plants, compared to plants affected by Fusarium wilt and anthracnose disease. In the present study the abundance of nematodes increased, even when the level of antioxidants and the growth variables increased. According to Polidoros et al. (2001), plants that over produce Catalase showed a decreased explain in resistance to pathogen infection, this could describein in explain in part the increased in the nematode infection, together with the increase of the antioxidant compounds. Hirt (2000) described that the increase of auxines in plants, increased the root formation, which could let more physical space for nematode and mycorrhizal settlement. Some studies have shown that the AMF inoculation could increase plant hormone production. In conclusion, the results from the present study demonstrate induction of tolerance in *I. balsamina* by AMF against the root knot nematode *M. incognita* despite of an increased level of nematode root infection.
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