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SHORT NOTE

Arbuscular mycorrhizal fungi affect both penetration and further life stage development of root-knot nematodes in tomato

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Abstract The root-knot nematode *Meloidogyne incognita* poses a worldwide threat to agriculture, with an increasing demand for alternative control options since most common nematicides are being withdrawn due to environmental concerns. The biocontrol potential of arbuscular mycorrhizal fungi (AMF) against plant-parasitic nematodes has been demonstrated, but the modes of action remain to be unraveled. In this study, *M. incognita* penetration of second-stage juveniles at 4, 8 and 12 days after inoculation was compared in tomato roots (*Solanum lycopersicum* cv. Marmande) precolonized or not by the AMF *Glomus mosseae*. Further life stage development of the juveniles was also observed in both control and mycorrhizal roots at 12 days, 3 weeks and 4 weeks after inoculation by means of acid fuchsin staining. Penetration was significantly lower in mycorrhizal roots, with a reduction up to 32%. Significantly lower numbers of third- and fourth-stage juveniles and females accumulated in mycorrhizal roots, at a slower rate than in control roots. The results show for the first time that *G. mosseae* continuously suppresses root-knot nematodes throughout their entire early infection phase of root penetration and subsequent life stage development.

Keywords Mycorrhiza-induced resistance · Biocontrol · *Meloidogyne incognita* · Nematode penetration · Life stage development · Tomato

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Introduction

Plant-parasitic nematodes are cosmopolitan pests with a wide host range, causing annual crop losses over $100 billion worldwide (Bird et al. 2008). The sedentary root-knot nematodes, *Meloidogyne* spp., are particularly damaging. Most cultivated crops are attacked by at least one root-knot nematode species and their wide host range is rivaled only by that of arbuscular mycorrhizal fungi (AMF), since it includes the majority of all flowering plants (Trudgill and Blok 2001). Nematode management is usually achieved by nematicide application, with the size of the nematicide market estimated to be around $800,000,000 for vegetables alone (Sikora et al. 2008). However, control options are becoming limited due to the withdrawal of many nematicides and soil fumigants because of environmental and health concerns, which emphasizes the need for alternative management strategies (Trudgill and Blok 2001; Sikora et al. 2008). The potential of AMF as biological control agents has been demonstrated against a range of soil-borne pathogens (reviews by Whipps 2004; St-Arnaud and Vujanovic 2007; Akhtar and Siddiqui 2008), including nematodes (Pinochet et al. 1996; Hol and Cook 2005; Elsen et al. 2008).

The *Meloidogyne* life cycle comprises a mobile soil phase, as motile second-stage juveniles hatch from eggs in the soil and penetrate host roots near the root tip (Curtis et al. 2009). Once inside, the nematodes migrate into the vascular cylinder, become sedentary and initiate a feeding site consisting of five to seven multinucleate giant cells (Abad et al. 2009). Nematodes will become increasingly swollen while undergoing three additional moults. Hyperplasia and hypertrophy of the cells surrounding the feeding site lead to the formation of the most typical symptom, the root galls, which seriously impair plant nutrient and water uptake. The giant cells serve as sole food source and by consequence their establishment and maintenance is essential for nematode life stage development and reproduction (Caillaud et al. 2008).

The modes of action of mycorrhiza-induced resistance against plant-parasitic nematodes remain largely unknown. Most experiments considering the biocontrol interaction between AMF and nematodes have merely observed the endpoints of infection, ignoring the actual interaction processes as if taking place in a black box (Hol and Cook 2005), although recently some advances have been made. The chitinase gene *VCH3* has been associated with mycorrhiza-induced resistance of grapevine roots against *Meloidogyne incognita*, since colonization by *Glomus versiforme* primed the expression of the gene throughout the root system in response to nematode infection (Li et al. 2006). In addition, root extracts from tomato plants colonized by *G. fasciculatum* reduced survival of *M. incognita* juveniles (Shreenivasa et al. 2007). For the migratory nematodes *Radopholus similis* and *Pratylenchus coffeae*, the systemic nature of the *G. intraradices*-induced reduction of nematode reproduction was demonstrated in banana (Elsen et al. 2008). Various AMF isolates have been used to investigate the interaction with plant-parasitic nematodes, with *Glomus mosseae* being the most widely applied species (Veresoglou and Rillig 2011). A recently performed meta-analysis by these authors highlighted that the choice of the AMF isolate can have a profound impact on the level of biocontrol.

In this study, another attempt is made to contribute towards unraveling the mechanisms taking place inside the black box, with a focus on nematode penetration and their subsequent life stage development in mycorrhizal plant roots. So far, these events in the nematode life cycle have mainly been explored in relation to resistant cultivars and non-host plants (Trudgill and Blok 2001). Penetration of *M. incognita* second-stage juveniles in resistant and susceptible cultivars is usually similar, while the further life stage development is slowed down. Decreased juvenile penetration on the other hand seems to be characteristic of non-hosts of root-knot nematodes (Ehwaeti et al. 1999). Interference in these stages of the nematode infection cycle can thus have significant implications for nematode management. Although based on different mechanisms, the reduction of nematode penetration and slowing down of their further life stage development in mycorrhizal plants might also make a significant contribution to nematode management.

Materials and methods

Biological materials

In both experiments, the tomato cultivar Marmande (*Lycopersicon esculentum* cv. Marmande) was grown under greenhouse conditions at an ambient temperature of 20–27°C and 75% relative humidity. The AMF *G. mosseae*, originally isolated from banana in Tenerife, Spain, and now kept as a greenhouse stock culture on sorghum, was applied as mycorrhizal inoculum at sowing of the test plants (Elsen et al. 2008). The inoculum consisted of rhizosphere soil from 6-month-old sorghum pot cultures containing spores, hyphae and heavily colonized root pieces. The sedentary root-knot nematode *M. incognita* was originally isolated from banana in Malaysia and afterwards maintained as a greenhouse stock culture on tomato cv. Marmande. When used for inoculation, egg masses were extracted from the tomato roots and freshly hatched second-stage juveniles were collected by using modified Baermann dishes (Hooper et al. 2005).

Nematode penetration experiment

Tomato plants were planted in the greenhouse in 1-l pots containing a non-sterilized substrate mixture of quarts river sand and potting soil (Brill Typical Type 3 'Topdressing grass') in a 2:1 ratio, a substrate shown previously to be compatible with a high nematode infection potential (Vos et al. 2011). For the mycorrhizal treatment, 200 ml of rhizosphere soil colonized by *G. mosseae* was added. Plants from the non-mycorrhizal control treatment received 200 ml of rhizosphere soil from non-colonized sorghum plants. All plants received 1 g of a slow-release fertilizer at planting (Substral Osmocote® controlled release fertilizer, NPK; 14-13-13). After 6 weeks, eight mycorrhizal plants were uprooted to determine mycorrhizal colonization by staining the roots with ink-vinegar (Vierheilig et al. 1998). After clearing, staining and destaining, 20 root pieces of 1 cm were mounted on slides and observed with a light microscope. The frequency of mycorrhizal colonization (*F*%) was calculated as the percentage of root segments colonized by hyphae, arbuscules or vesicles. In addition, the intensity of colonization (*I*%) was estimated as the abundance of hyphae, arbuscules and vesicles in each mycorrhizal root fragment (Plenchette and Morel 1996). After confirmation of

mycorrhizal colonization, plants were inoculated with 1,000 freshly hatched *M. incognita* second-stage juveniles. Penetration of the second-stage juveniles in control and mycorrhizal root systems was assessed at 4, 8 and 12 days after inoculation (DAI) by maceration-sieving of the roots (Speijer and De Waele 1997) followed by counting using a light microscope. Nematode penetration rate was calculated as the final nematode population (P_f) divided by the inoculated initial nematode population (P_i). Each treatment consisted of eight replications.

Nematode life stage development experiment

Preparation of tomato plants and assessment of mycorrhizal colonization was performed in the same way as for the penetration experiment, and plants were also inoculated with 1,000 freshly hatched *M. incognita* second-stage juveniles.

All life stages of the nematodes that penetrated the control or mycorrhizal roots were visualized by acid fuchsin staining of the roots (Byrd et al. 1983), followed by observation with a light microscope. The number of second-stage and third-stage juveniles, fourth-stage juveniles and females was counted in acid fuchsin stained roots at 12 DAI, 3 and 4 weeks after inoculation (WAI). At 12 DAI, tomato plants were transplanted to new substrate free of nematodes to avoid further second-stage juvenile penetration and to focus on further life stage development of the nematodes that had already penetrated the roots. Each treatment consisted of eight replications.

Statistical analysis

Nematode infection data of both experiments were statistically analyzed by analysis of variance (ANOVA) when the conditions for ANOVA were met (i.e., normal distribution and homogeneity of variances), using Statistica® software (Release 7, Statsoft, Tulsa, OK, USA). Tukey's HSD test was applied for multiple comparisons of group means. Prior to analysis, nematode numbers and penetration rates were log (x+1) or arcsin(x/100) transformed, respectively, to reduce data variance.

Results

Prior to nematode infection, establishment of the mycorrhizal colonization in both experiments was confirmed by the presence of hyphal structures, arbuscules and vesicles inside the stained tomato root cortices. Colonization frequency averaged 75%, with an intensity of 24%. Mycorrhizal colonization increased over the 4-week period of the nematode life stage development experiment, resulting in an average colonization frequency of 90% with an intensity of 32%. The plant parameters root and shoot weight did not differ significantly between mycorrhizal and control plants (data not shown).

Penetration of *M. incognita* second-stage juveniles in tomato roots increased significantly (P<0.05) from 4 up to 12 days after inoculation (DAI), with the penetration rate in the control plants increasing from 2% at 4 DAI up to 15% at 12 DAI (Fig. 1). Nematode penetration was significantly (P<0.05) lower in mycorrhizal roots at the three observation time points, with a penetration rate increasing from 1% at 4 DAI up to 10% at 12 DAI. The final penetration rate of the second-stage juveniles at 12 DAI was 32% less in mycorrhizal roots compared to control roots (Fig. 1).

In the nematode life stage development experiment, second- and third-stage juveniles were observed inside roots of mycorrhizal and control plants at 12 DAI, reaching a peak population at 3 weeks after inoculation (WAI) and decreasing again 1 week later. Overall, significantly (P<0.05) less second- and third-stage juveniles were observed in mycorrhizal roots compared to control roots at each observation time point, with the effect being most pronounced at 3 WAI. At this time point, the number of second- and third-stage juveniles was 77% lower in mycorrhizal roots compared to control roots (Fig. 2a). The next life stage, the fourth-stage juveniles, also appeared at 12 DAI in both mycorrhizal and control tomato roots, and reached a peak population at 4 WAI in the control roots while remaining low in the mycorrhizal roots. Significantly (P<0.05) less fourth-stage juveniles were present in mycorrhizal roots at 4 WAI, with a reduction of 94% compared to control roots (Fig. 2b). The adult female life stage was observed from 3 WAI on in control roots and peaked at 4 WAI, while females were not

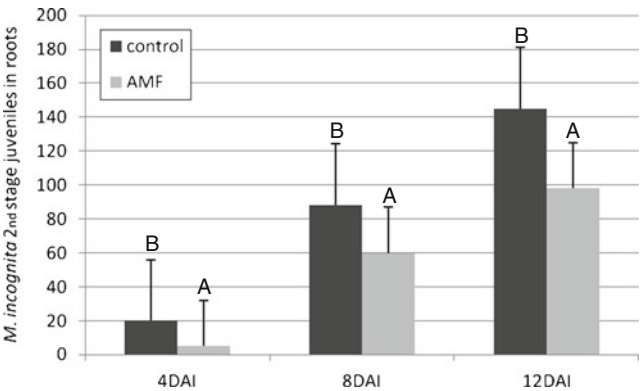

Fig. 1 Penetration of *Meloidogyne incognita* second-stage juveniles at 4, 8 and 12 days after inoculation (*DAI*) with 1,000 freshly hatched second-stage juveniles in tomato cv. Marmande roots without (control) or with *Glomus mosseae* (*AMF*). Data based on maceration-sieving of roots. Error bars represent standard error of eight replications. A two-way ANOVA was carried out on log(x+1) transformed data. Different letters indicate a significant difference (P≤0.05) of treatment (Control or AMF) according to the Tukey HSD test

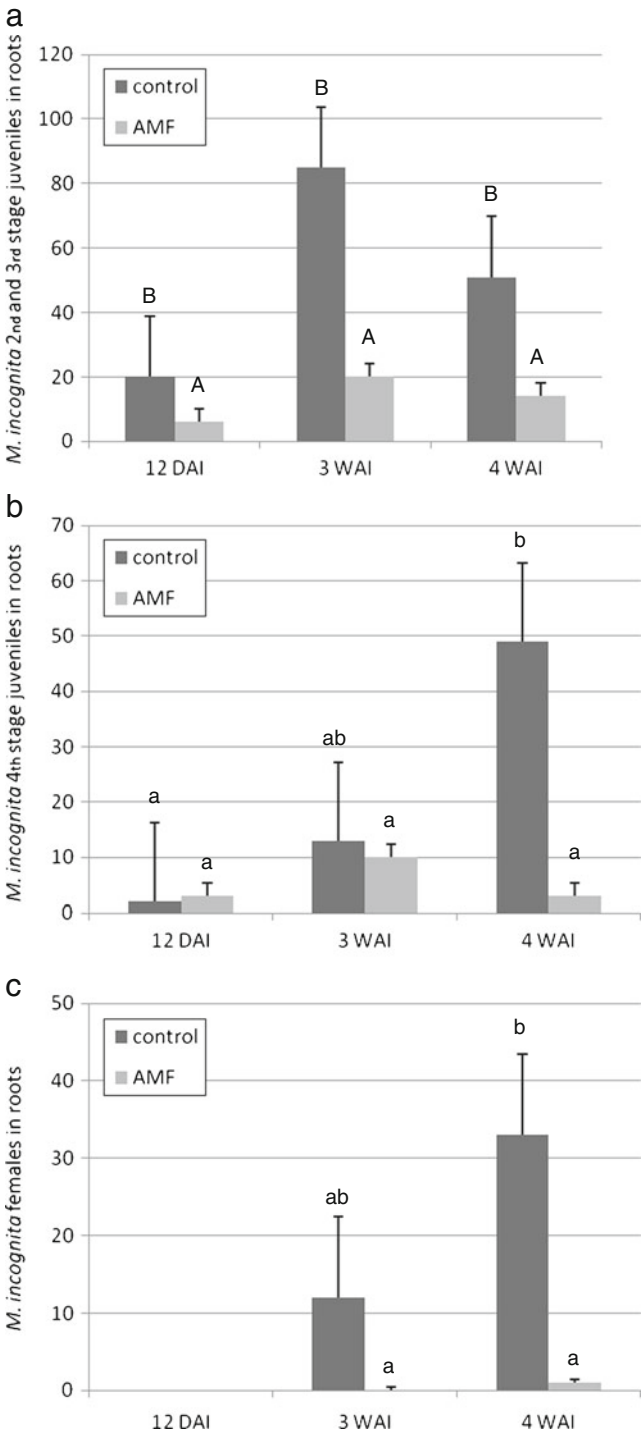

Fig. 2 Number of *Meloidogyne incognita* second- and third-stage juveniles (**a**), fourth-stage juveniles (**b**) and females (**c**) at 12 days (*DAI*), 3 and 4 weeks (*WAI*) after inoculation with 1,000 freshly hatched second-stage juveniles in tomato cv. Marmande roots without (control) or with *Glomus mosseae* (AMF). Data based on acid fuchsin staining of roots. Error bars represent standard error of eight replications. A two-way ANOVA was carried out on log(x+1) transformed data. Different capital letters or lowercase letters indicate a significant (P≤0.05) main effect of treatment (Control or AMF) or a significant (P≤0.05) interaction effect of treatment and time, respectively, according to the Tukey HSD test

yet observed in mycorrhizal roots at 3 WAI and appeared in low numbers at 4 WAI. Significantly (P<0.05) fewer females were observed in mycorrhizal roots at 4 WAI, with a reduction of 97% compared to the control roots (Fig. 2c).

Discussion

Penetration of tomato cv. Marmande roots by *M. incognita* second-stage juveniles was significantly lower in mycorrhizal roots compared to control roots. In the control roots, the penetration rate reached a maximum of 15% at 12 DAI, which is in accordance with previous penetration experiments under similar conditions for this nematode on tomato. In the report by Dababat and Sikora (2007), for example, *M. incognita* penetration rate varied from 8% to 15% between experiment repetitions.

For the nematode life stage development experiment, data were recorded up to 4 WAI. This corresponds to one life cycle of *M. incognita*, which usually takes 20 to 30 days to complete on average, depending on environmental conditions (Ploeg and Maris 1999). Once the second-stage juveniles have penetrated the roots, the life stage development of *Meloidogyne* species comprises three additional moults before reaching the adult life stage. It takes approximately 14 days before second-stage juveniles moult into third-stage juveniles, while fourth-stage juveniles usually emerge rapidly, and this phase typically lasts only 4 to 6 days (Moens et al. 2009). This was indeed reflected in the data of the nematode life stage development experiment, with the presence of third-stage juveniles at 12 DAI and the peak of fourth-stage juveniles lagging 1 week behind the peak of the previous life stage. The life stage development rate seemed slowed down in mycorrhizal roots. All subsequent life stages appeared in gradually lower numbers in the mycorrhizal roots compared to the normal life stage development in the control roots.

In summary, this report showed that less second-stage juveniles were able to enter mycorrhizal roots, and in addition, their development into the subsequent life stages was hampered compared to that in control roots. To our knowledge, this is the first report of a continuously suppressing effect of AMF on root-knot nematodes throughout their entire early infection phase of root penetration and subsequent life stage development. Reports about mycorrhiza-induced resistance against nematodes (Pinochet et al. 1996; Hol and Cook 2005; Akhtar and Siddiqui 2008) usually demonstrate an effect of mycorrhizal colonization on nematode infection after completion of several life cycles of the nematode, without assessing the contribution of nematode penetration or life stage development to the overall biocontrol effect. Akhtar and Siddiqui (2008) provide an overview of research papers on the interaction between

various AMF species and plant-parasitic nematodes, mainly of the genera *Meloidogyne* and *Heterodera*. Pre-establishment of the mycorrhizal symbiosis seems to be a general prerequisite for an effective reduction of nematode infection, as has also been shown for other pathogens (Slezack et al. 2000). The effect of AMF on nematodes is mostly demonstrated as a reduction in root galling or overall nematode reproduction (Akhtar and Siddiqui 2008). Few papers have specifically investigated nematode penetration, with various outcomes. Suresh et al. (1985) did not observe a difference in *M. incognita* penetration of *G. fasciculatum*-colonized tomato roots, while Mahanta and Phukan (2000) reported a reduction in *M. incognita* penetration of *G. fasciculatum*-colonized black gram, despite a short period for pre-establishment of the symbiosis. Reduced penetration of *M. incognita* second-stage juveniles has also been observed when other biocontrol agents than AMF were applied onto tomato roots. A significant reduction in juvenile penetration was demonstrated for example in the presence of endophytic bacteria (Munif et al. 2001) and of a non-pathogenic *Fusarium oxysporum* strain (Dababat and Sikora 2007).

The effect of AMF on nematode development has been investigated in terms of overall nematode reproduction (Jaizme-Vega et al. 1997), but not in terms of nematode life stage development. In this report, we focused on the infection phases that precede the nematode reproduction, namely both nematode penetration and further life stage development, and it seems indeed that lower juvenile penetration and subsequent life stage development are at least partially responsible for the longer generation time and lower overall nematode reproduction in *G. mosseae*-colonized plants.

The reduction in nematode penetration can have several causes and might, for example, result from an AMF effect on motility or host finding behaviour of the second-stage juveniles in the soil. In order to penetrate a root, nematodes first have to be able to guide themselves towards a suitable host and infection site, for which they rely on root exudation (Wuyts et al. 2006b; Curtis et al. 2009). Mycorrhizal symbiosis leads to both quantitative and qualitative differences in root exudation (Jones et al. 2004), including differences in amino acids, flavonoids, phenolic compounds, sugars and organic acids (Lioussanne et al. 2009). For several phenolic compounds, negative effects on nematode chemotaxis, motility or survival have been reported (Wuyts et al. 2006a). Recently, Vos et al. (2011) reported that the addition of root exudates originating from mycorrhizal tomato plants significantly decreased *M. incognita* root penetration. In addition, the second-stage juveniles were temporarily paralyzed in vitro when exposed to mycorrhizal root exudates.

Because of the differences in root exudation, the mycorrhizosphere harbours a different microbial community. The presence and abundance of facultative anaerobic bacteria, fluorescent pseudomonads, *Streptomyces* species and chitinase-producing actinomycetes may differ depending on the AMF species involved (Marschner and Baumann 2003; Harrier and Watson 2004) which might in turn have an antagonistic effect on the nematodes. For example, root exudates originating from mycorrhizal plants attracted PGPR like *Pseudomonas fluorescens* (Sood 2003) and stimulated other beneficial soil microorganisms like *Trichoderma*, which are both organisms known to exert a biocontrol effect on plant-parasitic nematodes (Dong and Zhang 2006; Sikora et al. 2008). The mycorrhiza-induced biocontrol can thus be enhanced by synergistic interactions with PGPR or other soil microorganisms (Lioussanne et al. 2009).

The nematodes that do reach a suitable infection site probably still face other hurdles. Cell wall fortifications in mycorrhizal plant roots might also be responsible for the lower nematode penetration observed. Dehne (1982) indeed observed increased lignification of root endodermal cells induced by mycorrhizal colonization and stressed the possible relevance in plant defence. In the case of tomato infection by the fungal pathogen *Phytophthora parasitica*, the role of cell wall modifications in biocontrol was demonstrated (Cordier et al. 1998). Plant cells containing *G. mosseae* were reinforced by callose, while the systemic defence response involved elicitation of host wall thickenings in reaction to intercellular hyphae from the pathogen. Vigo et al. (2000) also observed a reduction in *P. parasitica* penetration loci in mycorrhizal tomato roots. Other biochemical changes inside the mycorrhizal plant roots could also be responsible for the reduced penetration and further life stage development. Plant defence mechanisms are transiently activated in response to mycorrhizal symbiosis formation (Gianinazzi-Pearson et al. 2009), which triggers a primed state of jasmonate-dependent defence responses throughout the plant upon subsequent pathogen attack (Pozo and Azcon-Aguilar 2007), including the formation of phytoalexins, hydroxyproline-rich glycoproteins, phenolic compounds, peroxidases, chitinases, β-1,3-glucanases and PR proteins (Harrier and Watson 2004). Extracts of mycorrhizal tomato roots have been reported to reduce root-knot nematode survival, but the responsible compounds remain to be elucidated (Shreenivasa et al. 2007).

Despite numerous reports about the biocontrol potential of AMF, their actual use as biological control agents is still limited and a deeper insight into the working mechanisms can contribute to a better application strategy (Dong and Zhang 2006). Biocontrol applications could then be fine-tuned, for example by combining AMF with other possible biological control agents with a different mode of action. In addition, when it is known at which stadium of the nematode infection the mycorrhiza-induced resistance comes into play, we can proceed to more in-depth targeted molecular studies which could for example contribute to the discovery of marker genes for nematode resistance.

In conclusion, this study demonstrated for the first time a continuously suppressing effect of AMF on root-knot nematodes throughout their entire early infection phase of root penetration and subsequent life stage development. These effects are probably an important factor contributing to the overall mycorrhiza-induced resistance that has already been observed against root-knot nematodes. Penetration by second-stage juveniles was consistently lower in mycorrhizal roots, and their subsequent life stage development consistently slower. It remains to be determined to what extent effects on the other phases of the nematode life cycle play a role in the overall biocontrol: AMF might for example as well alter nematode behaviour in the soil. The molecular basis underlying the reduced nematode penetration and life stage development is not yet understood and merits further in-depth investigation.

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