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SHORT NOTE

Role of the modification in root exudation induced by arbuscular mycorrhizal colonization on the intraradical growth of *Phytophthora nicotianae* in tomato

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Abstract We studied the role of modification in root exudation induced by colonization with *Glomus intraradices* and *Glomus mosseae* in the growth of *Phytophthora nicotianae* in tomato roots. Plants were grown in a compartmentalized plant growth system and were either inoculated with the AM fungi or received exudates from mycorrhizal plants, with the corresponding controls. Three weeks after planting, the plants were inoculated or not with *P. nicotianae* growing from an adjacent compartment. At harvest, *P. nicotianae* biomass was significantly reduced in roots colonized with *G. intraradices* or *G. mosseae* in comparison to non-colonized roots. Conversely, pathogen biomass was similar in non-colonized roots supplied with exudates collected from mycorrhizal or non-mycorrhizal roots, or with water. We cannot rule out that a mycorrhiza-mediated modification in root exudation may take place, but our results did not support that a change in pathogen chemotactic responses to host root exudates may be involved in the inhibition of *P. nicotianae*.

Keywords Biocontrol · Root exudates · Arbuscular mycorrhizal symbiosis · *Phytophthora nicotianae* · *Solanum lycopersicum*

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Introduction

Phytophthora nicotianae is an Oomycete showing a huge host range, infecting more than 72 genera from 42 plant families (Erwin and Ribeiro 1996). This pathogenic species is at the origin of root rot diseases responsible for large yield losses in many important crops, including tomato. The attraction of *Phytophthora* zoospores and germ tubes towards roots and root exudates has been described (Carlile 1983; Deacon and Donaldson 1993) and constitutes a critical step in the onset of disease. Among soil microbial communities, the arbuscular mycorrhizal (AM) fungi have been shown to lower disease development in a wide range of plant–pathogen associations (St-Arnaud and Vujanovic 2007; Whipps 2004), including *P. nicotianae* infecting tomato plants colonized with *Glomus mosseae* BEG 12 (Cordier et al. 1996; Trotta et al. 1996; Vigo et al. 2000). AM fungi induction of cell defense responses were shown and suggested to be key factors limiting the pathogen proliferation within host roots (Cordier et al. 1998; Pozo et al. 2002), but how defense pathways are modulated during mycorrhizal colonization and with what importance they contribute in hampering pathogen growth are still poorly known (Pozo and Azcon-Aguilar 2007). AM colonization has also been shown to change the amount and quality of host root exudates (Azaizeh et al. 1995) and the chemotactic response of soil bacteria (Sood 2003). Recently, exudates from in vitro grown tomato roots colonized with *Glomus intraradices* DAOM 181602 were shown to modify the chemotactic response of *P. nicotianae* zoospores (Lioussanne et al. 2008).

Here, we tested the hypothesis that mycorrhizal colonization of tomato plants or application of root exudates from mycorrhizal plants would similarly affect the chemotactic response of *P. nicotianae* and hence root infection by this

pathogen, in non-axenic soil conditions. The objectives were (1) to test if *G. intraradices* DAOM 181602 reduces tomato root infection by *P. nicotianae* similarly to *G. mosseae* BEG 12 and (2) to compare the effect of direct inoculation with *G. mosseae* or *G. intraradices* with the effect of application of root exudates collected from tomato plants colonized with these AM fungal species onto the roots of non-mycorrhizal plants challenged with *P. nicotianae* on the intraradical biomass of this pathogen.

Materials and methods

Biological material and growth conditions

The growth substrate was a 2:2:1 (*v*/*v*) mix of field sandy loam, sand, and commercial potting mix containing humus, compost, perlite, and vermiculite (Tropical plant mix, Modugno-Hortibec, Montréal, Canada). The final soil mix (68% sand, 14% silt, 3% clay, 15.0% organic matter, 144 mg kg^{-1} of P, 412 mg kg^{-1} of K, 2,419 mg kg^{-1} of Ca, 421 mg kg^{-1} of Mg, pH5.63) was autoclaved twice for 60 min at 121°C. To reintroduce a microbial community exempt of AM fungi, a 500-g soil subsample was mixed for 30 min in 1.5 L of sterilized Milli-Q water, passed through Whatman No. 1 and 42 filters, and added to 5 kg of growth substrate. The mix was homogenized by hand using sterilized tools and gloves daily at 26°C for 2 weeks before use.

Leek (*Allium porrum* cv. Farinto) and tomato (*Solanum lycopersicum* cv. Cobra) seeds were surface-sterilized 15 min in 70% ethanol, followed by 20 min in 6% sodium hypochlorite plus 1% Triton X100 and rinsed three times in sterilized Milli-Q water. Seeds were germinated for 48–96 h on Tryptic Soy Agar (Quélab, Montreal, Canada) and transferred to the experimental units. After 4 days, seedlings were thinned to one per compartment and grown in a greenhouse with 16 h daylight (22–20°C). Plants were fertilized with 20 mL of 5× Long Ashton nutrient solution (Hewitt 1966) per week and watered with deionized water as needed.

Ri T-DNA-transformed *Daucus carota* roots colonized with *G. intraradices* (DAOM 181602) were grown as described in Fortin et al. (2002), in the dark at 26°C for 6 months. The spores were separated from the gel in sodium citrate buffer (Doner and Bécard 1991) and suspended in sterile water. *G. mosseae* (BEG 12) spore production and disinfection were performed as described in Budi et al. (1999) with modifications. Spores were recovered by wet sieving and decanting, and purification was carried out by two successive centrifugations at 3,000 rpm for 2 min in a density gradient with a 60% (*w*/*v*) sucrose layer at the bottom. Spores were collected from the gradient interface, thoroughly washed with sterile water and disinfected using a Buchner filtration system fitted with a Whatman No. 4 filter paper. The spores were suspended 30 s in 70% ethanol, rinsed three times in sterile water, immersed for 20 min in 20 g L^{-1} chloramine T plus one drop of Tween 80, rinsed ten times for 1 min in sterile water, and then incubated 24 h in Tryptic Soy Broth with 0.25 g L^{-1} ampicillin and 0.1 g L^{-1} streptomycin sulfate, at 24°C, in darkness. Finally, they were transferred to a new Buchner filtration system, rinsed in sterile water, incubated for 20 min in 20 g L^{-1} chloramine T and rinsed again ten times for 1 min before being suspended in sterile water. Viability of the spores was estimated by incubation in 1 g L^{-1} MTT for 72 h at 22°C (Walley and Germida 1995). They were cold-treated at 4°C for 2 weeks in sterilized distilled water prior to inoculation (Juge et al. 2002).

P. nicotianae (isolate 201) was grown on autoclaved V8 agar [V8 juice diluted 1:10 in Milli-Q water, 2 g L^{-1} $CaCO_3$, 10 g L^{-1} gellan gum, 0.05 g L^{-1} piramicin, 0.25 g L^{-1} ampicillin, 0.01 g L^{-1} rifampicin] at 26°C, under 16 h light per day (Tuite 1969). After 2 weeks, to inoculate each experimental unit, the mycelium from ten Petri dishes was scraped, mixed in 100 mL sterile water and blended two times for 4 s before use.

Experimental set-up, plant inoculation, and production of root exudates

In each experimental system (Fig. 1; see details below), 12 tomato plants were either submitted to the application of

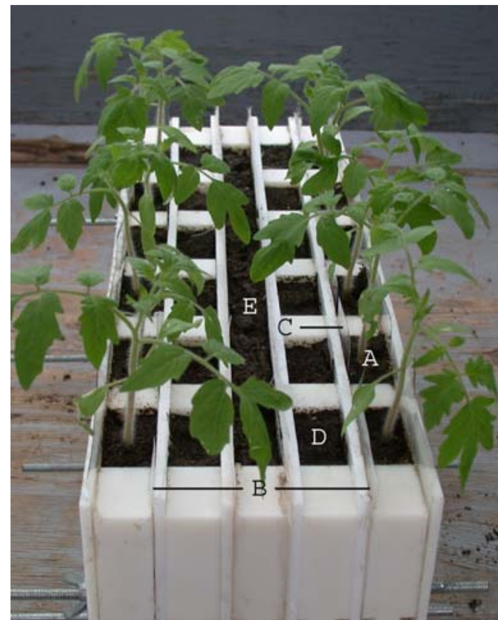

Fig. 1 Using a compartmentalized growth system, tomato plants were either submitted to the application of exudates from mycorrhizal tomato plants or to direct root colonization with an AM fungus, with the corresponding negative controls. Each experimental system comprised a full complement of the 12 treatment combinations. *A* Growth compartments with tomato plants, *B* 48-µm nylon membranes, *C* plastic sheets, *D* in-between unit, *E P. nicotianae*-infested unit

exudates from mycorrhizal tomato plants or to direct root colonization with an AM fungus, with the corresponding negative controls. Thus, half of the plants was supplied with sterilized pure water (E−) and either colonized with *G. intraradices* (Gi), *G. mosseae* (Gm), or not colonized (G−), while the other half received daily 2 mL of a standardized tomato root exudate solution collected from plants colonized with *G. intraradices* (E^{Gi}), *G. mosseae* (E^{Gm}), or not colonized (E^{G-}). After 3 weeks, plants were either inoculated (P+) or not (P−) with *P. nicotianae*. Therefore, each experimental system included the following treatment combinations: E−G−P−, E−GiP−, E−GmP−, E−G−P+, E−GiP+, E−GmP+, E^{G-}G−P−, E^{Gi}G−P−, E^{Gm}G−P−, E^{G-}G−P+, E^{Gi}G−P+, E^{Gm}G−P+ (Table 1). The experimental design was a split-plot with the six mycorrhizal inoculation/exudate application treatments randomized in the main plots, and *P. nicotianae* inoculation treatments randomized in the subplots of each experimental system. There were four blocks, each containing one experimental system with a full complement of treatment combinations, giving therefore four independent replicates per treatment.

A compartmentalized plant growth system based on Wyss et al. (1991) was used with modifications (Fig. 1). Using polyethylene plates, 50×16×2.5 cm units divided in six growth compartments were built with both sides covered with a 48 µm nylon membrane. To ensure a fast and homogenous mycorrhizal colonization (treatment Gi or Gm), one tomato seedling was transferred in each compartment and the unit was placed between two similar units containing leek plants previously colonized with *G. intraradices* on one side and *G. mosseae* on the other side. Units containing 2-week-old leek plantlets had previously been inoculated with a water suspension of 500 viable spores of *G. intraradices* or *G. mosseae* poured on the roots and grown for 7 weeks until use. Control uninoculated plants (G−) were obtained by insertion of a plastic sheet to prevent mycelium growth between compartments. Beginning 2 weeks after planting until harvest, 2 mL of tomato root exudates (collected from plants colonized with *G. mosseae*, *G. intraradices*, or not colonized) or sterilized Milli-Q water were poured daily on the soil of each compartment. In a second step, 3 weeks later, the units containing leek plants were taken away and replaced on one side by a *P. nicotianae*-infested unit prepared as follows: 12 tomato seedlings were planted and grown for 1 week, a 100-ml suspension of *P. nicotianae* mycelium was then evenly spread on the surface of the substrate and the infested plantlets were grown for 2 weeks before being cut to the soil level. An in-between unit containing only the substrate was added to create a gradient of exudates between plant compartments and the *P. nicotianae*-infested unit. Pathogen uninoculated control (P−) was obtained using a plastic sheet to prevent mycelium growth and zoospore swimming between compartments.

Root exudates of tomato plants colonized with *G. intraradices*, *G. mosseae*, or without mycorrhizal colonization were collected as described by Pinior et al. (1999). Briefly, tomato plants inoculated as described previously were grown for 5 weeks, the root system of each plant was

Table 1 Effect of AM fungal inoculation and root exudate application on mycorrhizal colonization and *P. nicotianae* biomass in tomato roots after 6 weeks of growth

Exudate application	AM fungi inoculation	Pathogen inoculation	AM root col. (%)[a]	*P. nicotianae* biomass[b]
Control	G−	P−	0.0±0.0	0.02±0.01
Control	Gi	P−	22.4±7.4 a	0.00±0.00
Control	Gm	P−	47.9±8.6 a	0.00±0.00
Control	G−	P+	0.0±0.0	0.33±0.10 a
Control	Gi	P+	24.5±5.8 a	0.04±0.03 b
Control	Gm	P+	41.9±8.0 a	0.00±0.00 b
E^{G-}	G−	P−	0.0±0.0	0.00±0.00
E^{Gi}	G−	P−	0.0±0.0	0.03±0.02
E^{Gm}	G−	P−	0.0±0.0	0.00±0.00
E^{G-}	G−	P+	0.0±0.0	0.32±0.07 a
E^{Gi}	G−	P+	0.0±0.0	0.27±0.11 a
E^{Gm}	G−	P+	0.0±0.0	0.59±0.33 a

Control plants supplied with pure water, E^{G-} plants supplied with exudates collected from plants not inoculated with an AM fungus, E^{Gi} and E^{Gm} exudates from plants colonized with *G. intraradices* or *G. mosseae*, *G−* plants not inoculated, *Gi* and *Gm* plants inoculated with *G. intraradices* or *G. mosseae*, *P−* plants not inoculated, *P+* plants inoculated with *P. nicotianae*

[a] Percentage of root length bearing mycorrhizal colonization±standard error of the mean. Non-inoculated plants were not colonized and not considered in ANOVA. Values are mean of four independent replicates

[b] Expressed in absorbance units from the ELISA test±standard error of the mean. Plants not inoculated with *P. nicotianae* showed nil values and were not considered in ANOVA. Values are mean of four independent replicates. Different letters mean a significant difference at $P<0.05$

washed under tap water and incubated in Erlenmeyer flasks filled with 100 mL sterilized Milli-Q water for 22 h. Solutions were sterilized by filtration with Whatman No. 4 and No. 42 filter papers and 0.22 µm nitrocellulose filters (Millipore) and lyophilized. Concentrations were adjusted to a ratio of 1 g of root fresh weight equivalent to 20 mL of exudate solution with sterile Milli-Q water. The pH was adjusted to 6.0 before being sterilized again with 0.22 µm nitrocellulose filters and solutions were kept at −20°C until use.

Plant harvest, measurements, and statistical analyses

After six weeks of growth, the substrate was withdrawn from the compartments containing tomato plants. The roots were harvested, cut in 1 cm sections, and mixed in sterilized distilled water. A first subsample was used for quantification of *P. nicotianae* using a commercial enzyme-linked immunosorbent assay (ELISA) kit (*Phytophthora* Pathoscreen kit, Agdia, Elkhart, IN, USA). Another subsample was used to assess mycorrhizal colonization after staining in black ink (Vierheilig et al. 1998), using the gridline intersect method (Giovannetti and Mosse 1980).

The effects of AM fungal inoculation (E−G−, E−Gi, E−Gm), exudate application (E^{G-}G−, E^{Gi}G−, E^{Gm}G−), and *P. nicotianae* inoculation (P+, P−) on root mycorrhizal colonization percentages and *P. nicotianae* biomass within tomato roots (quantified as OD values in ELISA tests) were analyzed by ANOVA using the Proc GLM procedure of SAS version 9.1.3. Normal distribution of residuals and homogeneity of variance assumptions were tested before ANOVA, and rank transformation of the ELISA OD units was performed before analysis to correct skewed distribution. A posteriori comparisons between means were performed using LSD tests.

Results

Mycorrhizal root colonization significantly reduced *P. nicotianae* intraradical growth ($P<0.05$) in comparison to non-colonized roots (Table 1). There was no significant difference in *P. nicotianae* biomass, measured with ELISA tests, between plants colonized with *G. mosseae* or *G. intraradices*. On the other hand, root exudates collected from mycorrhizal plants colonized with the same AM fungal species and applied to the root system of non-colonized plants had no effect on *P. nicotianae* growth. The ELISA OD values were significantly higher in roots treated with exudates collected from mycorrhizal plants than in roots inoculated with the AM fungi, and similar to the values measured in control plants supplied with exudates from non-mycorrhizal plants or sterilized pure water. As *P. nicotianae* was not detected in control plants not inoculated with the pathogen, data from P^- treatments were not included in the analysis of variance of *P. nicotianae*-OD units.

Mycorrhizal colonization of tomato roots was slightly higher ($P=0.08$) in plants inoculated with *G. mosseae* than with *G. intraradices,* with 45.0% and 23.4% of root length bearing mycorrhizal fungal structures, respectively. There was no effect of *P. nicotianae* inoculation on mycorrhizal root colonization and there was no significant interaction between AM fungi and *P. nicotianae* inoculation treatments (Table 1). As no mycorrhizal colonization was detected in control plants not inoculated with an AM fungus, data from G^- treatments were not included in the analysis of variance of percentages of root length bearing mycorrhizal colonization.

Discussion

In this experiment, root colonization with either *G. mosseae* or *G. intraradices* significantly reduced *P. nicotianae* growth within tomato roots, while the application of root exudates from plants colonized with the same AM fungal isolates did not modify the intraradical growth of this pathogen. Biocontrol of *P. nicotianae* in tomato roots colonized with the same *G. mosseae* strain was previously described (Cordier et al. 1996; Pozo et al. 2002; Trotta et al. 1996; Vigo et al. 2000). However, this is the first report of biocontrol induced by *G. intraradices* in tomato plants challenged with this pathogen, despite the study of Pozo et al. (2002) that contrarily reported no effect of another *G. intraradices* strain.

The lack of effect of the enrichment of non-mycorrhizal plant substrate with mycorrhizal root exudates suggests that modification in root exudation may not be involved in AM fungi-mediated biocontrol, as previously proposed (Norman and Hooker 2000; Vigo et al. 2000). We recently showed that exudates collected from actively growing Ri T-DNA transformed tomato roots colonized with *G. intraradices*, in vitro, were significantly more attractive for *P. nicotianae* zoospores than exudates from non-inoculated roots, while exudates from mature mycorrhizal roots were repulsive (Lioussanne et al. 2008). In the present study, exudates were collected from 5-week-old plants which perhaps did not present enough maturity to induce a significant repulsion of *P. nicotianae*. Moreover, the chemotactic responses of *P. nicotianae* within the soil may be different to those adopted by zoospores in vitro, since the soil solution is more complex. In addition, a soil filtrate was added to the autoclaved growth substrate in order to reintroduce a microbial community and this microflora could also have altered *P. nicotianae* behavior within the soil being antagonist to this pathogen capacity to infect plant roots. However, since *P. nicotianae* was detected in

root tissues of non-mycorrhizal plants supplied with water or with exudates, it was thus clearly able to grow from the *P. nicotianae*-infested unit towards tomato roots and to successfully infect plants. The presence of a soil microflora and the complexity of the soil substrate which were similar between treatments can therefore not account for the differential effect between direct mycorrhizal root colonization and that of mycorrhizal root exudates.

Here, the protocol used to prepare root exudates probably led to recovery of mainly hydrophilic molecules. Adsorption of specific molecules by the silt/clay and organic matter soil fractions can also not be ruled out and might explain the lack of effect of exudates from mycorrhizal roots on *P. nicotianae* infection. Nevertheless, using the same method of exudate preparation, Pinior et al. (1999) reported that exudates from *G. mosseae*-colonized cucumber plants contained molecules inhibiting further mycorrhizal colonization. This effect was later shown to be systemic as exudates collected from non-mycorrhizal roots of mycorrhizal plants induced comparable results (Vierheilig et al. 2003). If similar molecules are involved in the change of *P. nicotianae* zoospore chemotaxy observed in vitro, the absence of effects noticed here would therefore probably not be related to an inadequate exudate preparation protocol. Exudates from mycorrhizal plants or from AM fungal mycelium were also shown to impact sporulation and/or germination of other pathogens and soil microbes. Extracts from *G. intraradices* mycelium stimulated the growth of *Pseudomonas chlororaphis* and *Trichoderma harzianum*, had no effect on *Clavibacter michiganensis* and reduced conidial germination of *Fusarium oxysporum* f. sp. *chrysanthemi* (Filion et al. 1999). Root exudates from strawberry plants colonized by *Glomus etunicatum* or *Glomus monosporum* inhibited sporulation of *Phytophthora fragariae* compared to non-mycorrhizal plants (Norman and Hooker 2000). Contrarily, microconidium germination of *Fusarium oxysporum* f. sp. *lycopersici* was significantly increased by root exudates from *G. mosseae*-colonized tomato plants and 12 other species belonging to eight different plant families compared to non-mycorrhizal controls (Scheffknecht et al. 2007). This is the first report where the role of root exudates collected from mycorrhizal plants was assessed directly on plants challenged with a pathogen. Root exudates are involved in complex multitrophic interactions between plants and microorganisms (Bais et al. 2006) and exudates from mycorrhizal plants may as well impact the formation, the germination, and/or the attraction of other rhizosphere microbes in the soil. Those species may be specifically associated with microniches such as microbial biofilms around roots or mycorrhizal hyphae, and a general enrichment of the growth substrate such as performed here may have not accurately reproduced this complexity and mimic the effect of mycorrhizal colonization or the presence of the mycorrhizal network. Membrane filtering was recently shown to affect the bioactivity of root exudates presumably by retaining inhibitory compounds of microbial origin present in non-sterile exudates (Steinkellner et al. 2008). Such an effect might have also changed the impact of root exudates and could not be ruled out here.

In conclusion, root colonization with *G. mosseae* and with *G. intraradices* inhibited the intraradical growth of *P. nicotianae* in tomato while application of root exudates from tomato plants colonized with the same AM fungal species had no impact on the pathogen. We were not able to rule out that a modification in root exudation may be involved in the inhibition of *P. nicotianae* root infection, but our results did not support that a change in pathogen chemotactic responses to host root exudates may contribute to the biocontrol. It is likely that various mechanisms act simultaneously in this phenomenon, with their relative contribution varying with the combination of plant–pathogen–AM fungus species and environmental conditions. Stimulation of plant defense mechanisms and antagonistic mycorrhiza-associated bacteria have already been shown to occur with *G. mosseae* in this system. However, the sequence of events governing the modifications in the mycorrhizosphere microbial communities that may affect pathogen proliferation before infection remain to be identified to reach the full potential of the AM symbiosis in crop management.

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