Use of Root Organ Cultures To Investigate the Interaction between *Glomus intraradices* and *Pratylenchus coffeae*

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The interaction between *Glomus intraradices* and *Pratylenchus coffeae* on transformed carrot roots was studied in root organ culture. *G. intraradices* provided the roots with increased protection against *P. coffeae* by suppressing nematode reproduction in the roots. The internal and external mycorrhizal development was not influenced by the presence of the nematodes.

The effects of arbuscular mycorrhizal fungi (AMF) on damage caused by root nematodes have been widely addressed (2, 21, 23). However, most research so far has been conducted under greenhouse conditions, with potentially misleading results due, in part, to the presence of undesirable contaminants (3). In recent years, the development of the root organ culture (ROC) system has opened new avenues to study various aspects of the symbiosis (for a review, see reference 13). Numerous studies have been dedicated to interactions between AMF and bacteria and fungi (3, 4, 12, 18), while interactions with nematodes were seldom considered. One recent study reported the effect of AMF on the reproduction and root infestation of the nematode *Radopholus similis* by using this ROC system (10), but no data reported the impact of this nematode on the fungus development within and outside the host root. Since both organisms are obligatorily dependent on the root for the completion of their life cycle and occupy the same ecological root niche, each may impact the development of the other, either directly or indirectly. This communication reports, for the first time, the development and interaction of the obligatory parasitic nematode, *Pratylenchus coffeae*, and the obligatory AMF symbiont, *Glomus intraradices*, under ROC conditions.

Transformed carrot roots (*Daucus carota* L.) colonized with *G. intraradices* Schenck and Smith (MUCL 41833) and nonmycorrhizal transformed carrot roots were purchased from GINCO (Louvain-la-Neuve, Belgium). Both materials were provided in petri dishes on the modified Strullu-Romand (MSR) medium (reference 7, modified from the description in reference 25). The petri dishes were maintained in an inverted position in the dark at 27°C. To obtain enough transformed carrot roots for the experiment, the root cultures were multiplied by transferring individual 70-mm-long root tips to new petri dishes containing MSR medium every 3 weeks. The AMF cultures were incubated for 5 months in order to obtain enough AMF spores for the inoculation. Several thousand

spores were produced within this period and isolated by solubilization of the MSR medium (8), previous to the experiment.

A *P. coffeae* population from Ghana (originally isolated from *Musa* spp.) was maintained on *Medicago sativa* L. callus, grown on modified White's medium (11). Nematodes were extracted from the callus with a modified Baermann funnel (15).

At the beginning of the experiment, 48 90-mm-diameter petri dishes were filled with 40 ml of MSR medium. In each petri dish, one 70-mm-long transformed carrot root was transferred on the medium. Subsequently, they were separated in two homogenous groups (i.e., two groups of 24 petri dishes, each containing one transformed carrot root). One group was inoculated with approximately 100 spores of *G. intraradices*, while the other group received no inoculum.

The petri dishes were maintained horizontally but inverted at 27 ± 1°C in the dark for 5 weeks. At the end of this phase, both groups were further separated in two homogenous groups (i.e., four groups of 12 petri dishes in total). Subsequently, 12 petri dishes containing mycorrhizal roots and 12 petri dishes containing nonmycorrhizal roots were inoculated with 25 nematodes. Mature females were collected individually with a sterile micropipette and placed in a drop of sterile water near the root tips. The petri dishes were then identically maintained for 12 weeks. The experiment was arranged as a completely randomized design with the mycorrhizal or nonmycorrhizal carrot roots inoculated or not with the nematodes (four treatments in total). At the end of the experiment, 8 to 12 replicates were considered per treatment.

Root length and spore numbers were estimated under a binocular microscope at regular intervals (after 1, 2, 3, 5, 7, 9, 13, and 17 weeks), while extraradical hyphal length was estimated at the end of the experiment (week 17). The root length of the nonmycorrhizal and mycorrhizal treatments inoculated with nematodes and the mycelium length of the mycorrhizal treatments were measured using the formula of Newman (20). For the treatments containing AMF, the spores were counted individually (6). The treatments containing nematodes were examined for nematode reproduction. Nematodes were extracted from chopped root pieces and medium separately, using maceration-sieving (24). Numbers of juveniles, females, and males were determined by counting three 2-ml aliquots from a 200-ml homogenized suspension. For the AMF, the

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FIG. 1. Spore production by *G. intraradices* in MSR medium in the presence and absence of *P. coffeae*. Arrow, time of nematode inoculation; bars, standard error ($P \leq 0.05$).

frequency and intensity of root colonization was estimated after staining (16) by the method described by Declerck et al. (6).

Nematode populations and spore counts were $\log(x + 1)$ transformed, while frequency and intensity of mycorrhizal colonization were $\arcsine(x/100)$ transformed. The data that were normally distributed and had homogeneous variances were subjected to analysis of variance (19, 26), and the means were further separated by the Tukey test ($P < 0.05$) with the Statistica package (1).

The root length increased with time, whatever the treatment, and reached a plateau towards the end of the experiment (data not shown). At this time, i.e., week 17, the root length was 264 ± 198 cm in the nonmycorrhizal roots inoculated with nematodes and 177 ± 22 cm in the mycorrhizal roots inoculated with nematodes. Both values did not differ significantly between treatments.

No differences in sporulation dynamics were observed between the treatments without and with nematodes (Fig. 1). At the time of nematode inoculation, i.e., 5 weeks after mycorrhizal association, the average spore number per petri dish was approximately 6,500 for both treatments and increased to up to approximately 17,000 spores by the end of the experiment. No difference in mycelium length was observed at the end of the experiment. Averages of 1,052 and 1,064 cm of mycelium were measured in the absence and presence of *P. coffeae*, respec-

tively. A good internal mycorrhizal colonization was observed in both treatments. Frequencies of root colonization were 84 and 93% in the presence and absence of *P. coffeae*, respectively, while intensities of root colonization were 26 and 33% for both these two treatments, respectively. However, no statistically significant differences ($P \leq 0.05$) were found for these two parameters between the mycorrhizal roots grown in the presence and absence of *P. coffeae*.

The numbers of nematodes extracted from carrot roots and MSR medium are listed in Table 1. Penetration of roots and reproduction were observed in both the nonmycorrhizal and mycorrhizal treatments. All vermiform developmental stages were recovered from both roots and medium. The reproduction ratio was significantly lower in the mycorrhizal treatment than in the nonmycorrhizal treatment. In the presence of the AMF, the *P. coffeae* population was reduced by almost 50% compared to the nonmycorrhizal treatment. However, this reduction was not significant for all developmental stages. The number of females was reduced only in the roots. There were no significant differences in the number of juveniles and males in either roots or medium. Overall, the impact of the AMF on nematode population density was more pronounced in the roots than in the medium.

Both AMF and nematodes were able to complete their life cycles in the ROC system. For *G. intraradices*, a dense hyphal

TABLE 1. Nematode population densities in mycorrhizal and nonmycorrhizal transformed *D. carota* roots and MSR medium, 12 weeks after inoculation with 25 *P. coffeae* females^a

Root type	No. of nematodes in roots				No. of nematodes in medium				P_f^b	R_r^b
	Juveniles ^c	Females ^b	Males ^c	Total ^b	Juveniles ^c	Females ^c	Males ^c	Total ^c		
+AMF	181	415	113	709	25	189	111	325	1,034	41
-AMF	253	1,477	280	2,010	47	265	202	514	2,524	101

^a +AMF, mycorrhizal roots; -AMF, nonmycorrhizal roots; P_f , final population in roots and medium; R_r , reproduction ratio. All nematode population densities were $\log(x + 1)$ transformed prior to analysis.

^b Significant difference according to the Tukey test ($P \leq 0.05$).

^c No significant difference according to the Tukey test ($P \leq 0.05$).

network with numerous spores and high mycorrhizal root colonization were found at the end of the experiment. For *P. coffeae*, penetration and reproduction within the roots were observed in both the nonmycorrhizal and mycorrhizal treatments. Nematode damage in the roots was observed in all carrot roots inoculated with these nematodes as root necrosis, indicated by the yellowing-browning of the root system.

Neither the internal mycorrhizal root colonization nor the spore production and sporulation dynamics, as described by many researchers (5, 7), appeared to be influenced by the presence of the nematodes inside and outside the carrot roots. Therefore, it can be concluded that the nematode infection had no visual influence on the intraroot and extraradical development of the AMF under ROC conditions. In contrast, the results demonstrated unambiguously that *G. intraradices* grown under ROC conditions can provide transformed carrot roots with increased protection against *P. coffeae*. This was demonstrated by a smaller nematode population within the mycorrhizal roots, as already shown for *R. similis* (10). The suppressive effect of the AMF was more pronounced in the roots, where the nematodes feed and reproduce, than in the medium. The females in the roots were significantly suppressed, while both juveniles and males tended to be suppressed.

The mechanisms governing the reduction of the nematode populations observed in our experiment remain speculative. Improved nutrient status, microbial changes in the rhizosphere, competition for penetration sites and nutrients, biochemical changes in plant physiology and anatomical changes in the roots have been proposed as possible antinematode mechanisms (17). Hypotheses such as those involving microbial changes in the rhizosphere could reasonably be discarded, since the ROC is devoid of any undesirable microorganism. Competition for penetration and/or nutrient sites appeared more realistic, according to the marked decrease in nematode population observed in the mycorrhizal roots versus the non-mycorrhizal ones and the significantly larger suppressive effect observed in the mycorrhizal roots versus the medium. However, this hypothesis could not be supported due to the absence of correlations between nematode population densities within the root as well as in the medium and the internal AMF root colonization, external hyphal development, or spore production. Other factors, such as biochemical and/or anatomical changes in the mycorrhizal root, may be involved in the protection of the root against nematodes. Such hypotheses were proposed for the suppressive effect observed in mycorrhizal-transformed carrot roots against the fungus *Fusarium oxysporum* f. sp. *chrysanthemi* (3). This protection was associated, to a certain extent, with the accumulation of newly formed plant products at the site of infection, as corroborated by the results of Simoneau et al. (22), who observed an accumulation of symbiosis-related proteins in transformed tomato roots. Some of these proteins were thought to be involved in plant defense (14). Induction of lipid transfer protein and the phenylalanine ammonia lyase following mycorrhizal establishment within the root was further suggested (5) to explain plant defense response against pathogenic microorganisms. A systematic histological and cytological study, together with biochemical analysis, is required to further test this hypothesis as one possible mechanism for reduced susceptibility against *P. coffeae*.

Although the ROC system has several limitations (e.g.,

the absence of photosynthetic tissue, normal hormone balance, and physiological source-sink relationships), there are still many legitimate reasons to use this system to study the AMF-nematode interaction. The AMF form typical colonization structures, i.e., appressoria, arbuscules, and vesicles, produce profuse extraradical mycelium and spores, and are completing their life cycle. The early colonization occurs in a way similar to that under in vivo conditions (13). The nematode *P. coffeae* can infect and reproduce in the roots and cause damage in the in vitro roots that is similar to that in the in vivo roots. These findings suggest that the protection enhanced by AMF in ROC is not specific, as the reproduction of two different nematode species (i.e., *P. coffeae* and *R. similis*) was suppressed under these conditions. In addition, the effects of the interaction (suppression of the nematode population in the roots and no effect on the internal mycorrhizal development) reflect those observed in vivo (9). Moreover, the impact of the nematodes on the development of the external mycorrhizal mycelium could be studied for the first time. Therefore this ROC system may represent a valuable tool for studying the AMF-nematode interaction, complementary to classical experimental approaches. While the mechanisms involved in the nematode population reduction were not elucidated, this study supports the potential of the AMF ROC system for isolating factors (like biochemical changes in the mycorrhized roots and qualitative and quantitative changes in root and/or AMF exudates) involved in the interaction between nematodes and AMF. In addition, a hypothesis like microbial changes in the rhizosphere can be ruled out under ROC conditions.

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REFERENCES

1. Anonymous. 1997. Statistica. Statsoft, Tulsa, Okla.
2. Azcón-Aguilar, C., and J. M. Barea. 1996. Arbuscular mycorrhizas and biological control of soil-borne plant pathogens: an overview of the mechanisms involved. *Mycorrhiza* **6**:457-464.
3. Benhamou, N., J. A. Fortin, C. Hamel, M. St. Arnaud, and A. Shatila. 1994. Resistance response of mycorrhizal Ri T-DNA transformed carrot roots to infection by *Fusarium oxysporum* f. sp. *chrysanthemi*. *Phytopathology* **84**: 958-968.
4. Biancotto, V., D. Minerdi, S. Perotto, and P. Bonfante. 1996. Cellular interactions between arbuscular mycorrhizal fungi and rhizosphere bacteria. *Protoplasma* **193**:123-131.
5. Blilou, I., J. A. Ocampo, and J. M. Garcia-Garrido. 2000. Induction of LTP (lipid transfer protein) and PAL (phenylalanine ammonia lyase) gene expression in rice roots by the arbuscular mycorrhizal fungus *Glomus mosseae*. *J. Exp. Bot.* **51**:1969-1977.
6. Declerck, S., D. G. Strullu, and C. Plenchette. 1996. In vitro mass production of the arbuscular mycorrhizal fungus *Glomus versiforme*, associated with Ri T-DNA transformed carrot roots. *Mycol. Res.* **100**:1237-1242.
7. Declerck, S., D. G. Strullu, and C. Plenchette. 1998. Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. *Mycologia* **90**:579-585.
8. Doner, L. W., and G. Bécard. 1991. Solubilization of gellan gels by chelation of cations. *Biotechnol. Tech.* **5**:25-29.
9. Elsen, A. 2002. Study of the interaction between arbuscular mycorrhizal fungi and plant-parasitic nematodes in *Musa* spp. Ph.D. dissertation. Katholieke Universiteit Leuven, Leuven, Belgium.
10. Elsen, A., S. Declerck, and D. De Waele. 2001. Effects of *Glomus intraradices* on the reproduction of the burrowing nematode (*Radopholus similis*) in dioxenic culture. *Mycorrhiza* **11**:49-51.
11. Elsen, A., K. Lens, D. T. M. Nguyen, S. Broos, and D. De Waele. 2001. Development of aseptic culture systems of *Radopholus similis* for in vitro host-pathogen studies. *J. Nematol.* **33**:147-151.
12. Filion, M., M. St. Arnaud, and J. A. Fortin. 1999. Direct interaction between

- the arbuscular mycorrhizal fungus *Glomus intraradices* and different rhizosphere micro-organisms. *New Phytol.* **141**:525–533.
13. Fortin, J. A., G. Bécard, S. Declerck, Y. Dalpé, M. St. Arnaud, A. P. Coughlan, and Y. Piché. 2002. Arbuscular mycorrhiza on root-organ cultures. *Can. J. Bot.* **80**:1–20.
 14. Harrison, M. J., and R. A. Dixon. 1993. Isoflavonoid accumulation and expression of defence gene transcripts during the establishment of vesicular-arbuscular association in roots of *Medicago truncatula*. *Mol. Plant Microbe Interact.* **6**:643–654.
 15. Hooper, D. J. 1990. Extraction and processing of plant and soil nematodes, p. 45–68. *In* L. Luc, R. A. Sikora, and J. Bridge (ed.), *Plant parasitic nematodes in subtropical and tropical agriculture*. CAB International, Wallingford, United Kingdom.
 16. Koske, R. E., and J. M. Gemma. 1989. A modified procedure for staining roots to detect VA mycorrhizas. *Mycol. Res.* **92**:486–505.
 17. Linderman, R. G. 1994. Role of VAM fungi in biocontrol, p. 1–25. *In* F. L. Pfeleger and R. G. Linderman (ed.), *Mycorrhizae and plant health*. The American Phytopathological Society, St. Paul, Minn.
 18. McAllister, C. B., J. M. Garcia-Garrido, A. Garcia-Romera, A. Godeas, and J. A. Ocampo. 1996. In vitro interaction between *Alternaria alternata*, *Fusarium equiseti* and *Glomus mosseae*. *Symbiosis* **20**:163–174.
 19. Neter, J., W. Wasserman, and M. H. Kutner. 1990. Applied linear statistical models. Regression, analysis of variance and experimental designs. Irwin, Boston, Mass.
 20. Newman, E. I. 1966. A method of estimating the total length of root in a sample. *J. Appl. Ecol.* **3**:139–145.
 21. Pinochet, J., C. Calvet, A. Camprubi, and C. Fernandez. 1996. Interactions between migratory endoparasitic nematodes and arbuscular mycorrhizal fungi in perennial crops: a review. *Plant Soil* **185**:183–190.
 22. Simoneau, P., N. Louisy-Louis, C. Plenchette, and D. G. Strullu. 1994. Accumulation of new polypeptides in Ri T-DNA transformed roots of tomato (*Lycopersicon esculentum*) during the development of vesicular-arbuscular mycorrhizae. *Appl. Environ. Microbiol.* **60**:1810–1813.
 23. Smith, G. S. 1987. Interactions of nematodes with mycorrhizal fungi, p. 292–300. *In* J. A. Veech and D. W. Dickson (ed.), *Vistas on nematology*. Society of Nematologists, Hyattsville, Md.
 24. Speijer, P. R., and D. De Waele. 1997. Screening of *Musa* germplasm for resistance and tolerance to nematodes. International Network for the Improvement of Banana and Plantain (INIBAP) Technical Guidelines. INIBAP, Montpellier, France.
 25. Strullu, D. G., and C. Romand. 1986. Méthode d'obtention d'endomycorhizes à vésicules et arbuscules en conditions axéniques. *C. R. Acad. Sci., Paris* **303**:245–250.
 26. Winer, B. J. 1971. Statistical principles in experimental design. McGraw-Hill, New York, N.Y.