Microscopical examination of plant reaction in case of infection with *Trichoderma* and *Mycorrhizal* fungi

Accepted, Novemb

MIHAELA ENE[1], MIOARA ALEXANDRU[1]
[1]*University of Bucharest, Faculty of Biology, Department of Botany, Bucharest, Romania*
;

Abstract

Trichoderma spp. are active ingredients in a variety of commercial biofungicides used to control a range of economically important aerial and soil borne fungal plant pathogens. The antagonistic activity of biocontrol Trichoderma strains is attributable to one or more complex mechanisms, including nutrient competition, antibiosis, the activity of cell wall-lytic enzymes, induction of systemic resistance, and increased plant nutrient availability.

Mycorrhizal fungi form mutualistic symbioses with a vast majority of land plants, possibly more than 80 % of all land plants. Mycorrhiza represents an important group because they have a wide distribution; may contribute significantly to microbial biomass and to soil nutrient cycling processes in plants. The mycorrhizal fungi are part of biofertilizers, recognized for their beneficial effects: improved plant nutrition, soil fertility improvement, root pest and disease control, improved water usage, amelioration of toxic effects in soils.

The objective in this study is to analyze macroscopically and microscopically the interaction between Trichoderma sp. – mychorrizal fungi and plants in a tripartite system, in order to investigate the host behavior of the plant, as well as aspects concerning the interaction between the three organisms.

Keywords: Mycorrhizae, *Trichoderma*, Microscopical analysis, Defense reaction

Introduction

Among the microorganisms often used in the composition of biofertilizers, the different species of the saprophytic ***Trichoderma*** fungus are well-known fungal biocontrol agents. *Trichoderma* is a filamentous fungus that is widely distributed in the soil, plant material, decaying vegetation and wood [1].

Other fungi frequently used in the composition of biofertilizers are Arbuscular Mycorrhizae Fungi (AMF). They colonize plant roots and extend the root system into the surrounding soil. AMF are fungi that have developed a symbiotic (mutually beneficial) relationship with the root systems of living plants, from garden vegetables all the way up to old trees. By attaching to the feeder roots, mycorrhizae greatly extend the effective absorbing area available to plants. Research demonstrates that mycorrhizal filaments can explore volumes of soil hundreds to thousands of times greater than roots themselves. This relationship is beneficial because the plant enjoys improved nutrient and water uptake. AMF are chiefly responsible for **phosphorus (P) uptake** – the plants may be able to use insoluble sources of P when inoculated with mycorrhizal fungi but not in the absence of inoculation [2] - and early inoculation at the seedling stage has been proven beneficial. In addition, the relationship provides more uniform growth, increased leaf size, more flowering and increased vegetable yields. Networks of mycorrhizae filaments also gather abilities for protecting the plant from disease.

Therefore, the opportunity of using a biofertilizer combining the two categories of fungi is analyzed below. The interaction between each one and the host plant, as well as the interaction between the two fungi are investigated and discussed.

Materials and Methods

Soil-based substrate was prepared by mixing 2 parts of sand with 1 part of soil, pH 5-6,5, (AGRO CS" - www.agrocs.cz), followed by sterilization with gamma radiations. The purpose of soil mixing was to reduce the total phosphorus content, as well as loosening the substrate. The initial composition of the soil was as follows: P_2O_5 – 100-200 mg / liter, N - 150-250 mg / liter, K_2O - 200-300 mg / liter.

The gamma irradiation was performed in an industrial plant, with a ^{60}Co source. The exposure gave an absorbed dose of 15 KGy. The treatment aimed to eliminate the nematodes and competitive fungi.

Small potato tubercles were planted into gamma sterilized substrate and simultaneous infection was applied with both *Trichoderma spp.* (3 different strains) and mycorrhizal fungi. Ten replicates were prepared for every infection combination. Also, 10 replicates of mock-inoculated plats were associated (control). All fungi spores were homogenized in the substrate.

Microorganism(s) strains used were as follows:

- *Trichoderma longibrachiatum* (MUCL 20461), *Tr. virens* (MUCL 34687), *Tr. koningii* (MUCL 42800) – all conidial spores, ~75 UFC / g substrate.

- 8 different strains of Arbuscular Mycorrhizal Fungi (AMF): *Glomus intraradices*, *Glomus mosseae*, *Glomus aggregtum*, *Glomus monosporum*, *Glomus brazilianum*, *Glomus clarum*, *Glomus deserticola* and *Gigaspora margarita* – extraradicular spores, ~30 spores / g subtrate. The selected mycorrhizae fungi are well-suited to a variety of soils, climates and plants, and were present in the commercial product Mycorrhizae Root Dip Gel", from *Mushroom Research Center GmbH*, Austria.

For the *Trichoderma* strains, final spore concentration in the substrate was assessed by colony counting on PDA medium (Potato-Dextrose Agar, Merck), following a total bioburden test procedure.

All plants developed in a protected location, under natural light conditions. Temperature varied between 25 and 28^{o}C; all pots were light protected.

After 3 weeks of co-culturing, all growing parameters were measured and roots were harvested for microscopy. All 10 replicates were considered. Three roots from every plant were analyzed under a Zeiss (Axio-Imager.D1m) microscope; the thickest roots were chosen. From every parameter, average between replicates was made (see table 1.)

For microscopically analysis, classical trypan-blue [3] and cotton blue [4] staining protocols for fungi were applied, following a clearing in KOH solution. Also, some hyphae can easily be seen without any staining, in fresh preps.

Results and Discussions

Microscopical analysis showed a successful infection for all fungi strains. Ungerminated spores belonging to both groups of fungi have also been found (fig. 8).

In general, plant growth parameters were visible enhanced in the presence of AMF and *Trichoderma* fungi, comparing with control plants (see table 1).

Trichoderma longibrachiatum was most effective in terms of root length colonization as well as growth parameters (fig. 9). The infection is characterized by the development of hyphae coiled around the root and penetration of the radicular cortex (fig. 5, 6, 7).

AMF spores located in the near vicinity of the root germinated and infected the cortex, producing vesicles (fig. 2, 4). Numerous hyphae and arbuscules were observed along the root (fig. 1).

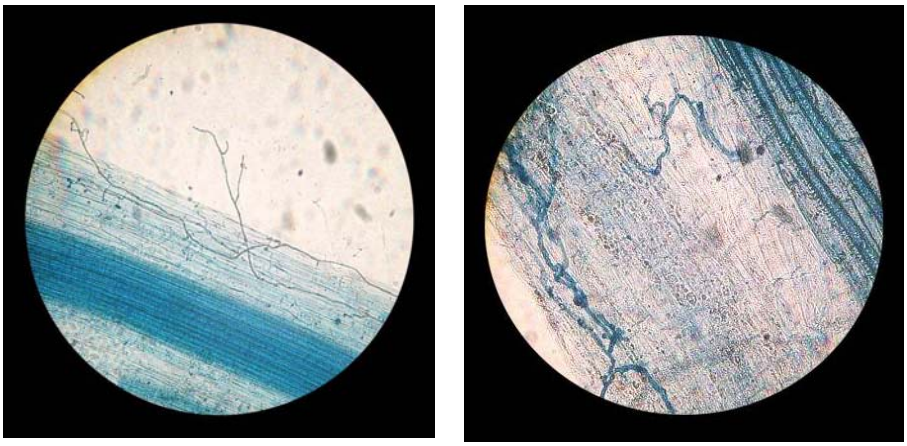


Figure 1. Intraradicular hyphae of AMF along and emerging from the root. Trypan-blue staining.

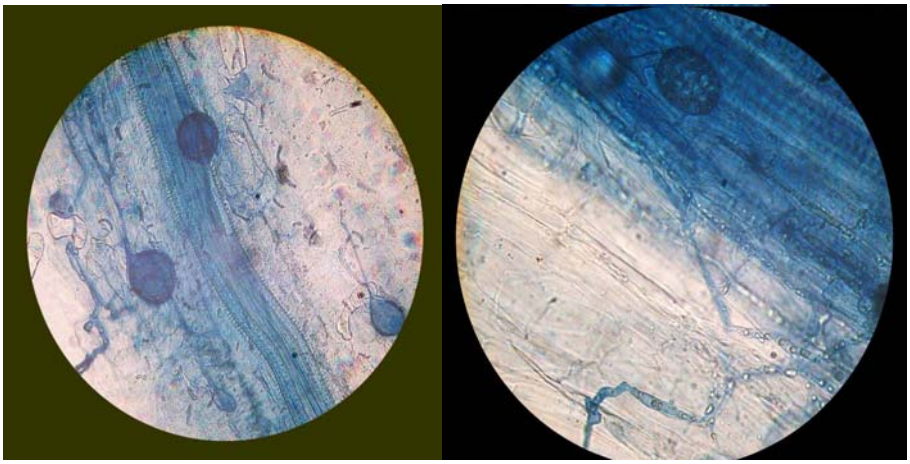


Figure 2. Intraradicular vesicles of AMF. Trypan-blue staining.

No area was found where the two species shared the root space. Moreover, in most cases, *Trichoderma* and AMF colonized distinct radicles. One single case was found where the 2 fungi infected the same root, still far away one from the other; the AMF colonized the root tip (about 2 cm above the tip), while the *Trichoderma* infected the superior part of the root.

Although *Trichoderma* is known as a mycoparasitical fungi (this is one explanation for its biocontrol effect), no parasitic interaction were observed over the AMF spores.

Different type of interactions between *Trichoderma* and AMF when infecting the same plant has been reported. Similar with our observations, Masadeh et al. [5] note that there was no evidence of negative interactions between the two beneficials with regard to AMF root colonization or population development of *T. viride* in the rhizosphere.

Figure 3. Extraradicular spore of AMF. No staining.

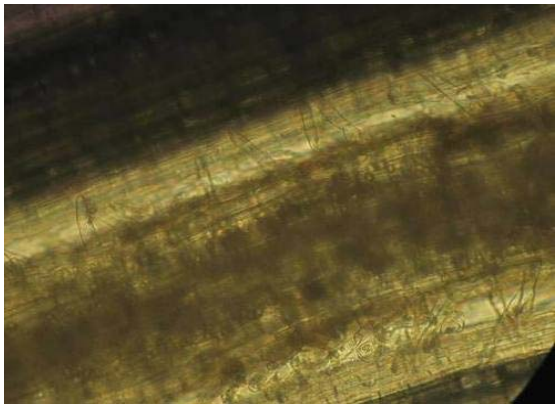
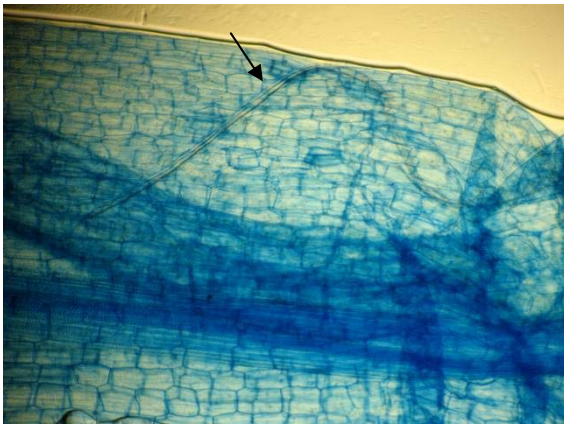


Figure 4. Intraradicular vesicles of AMF. Fresh prep from a young, squashed root. No staining.



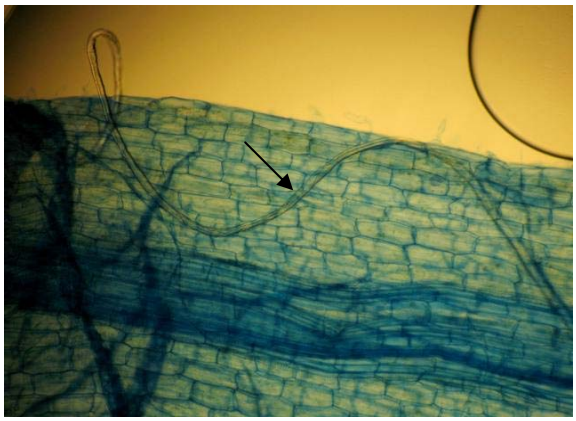
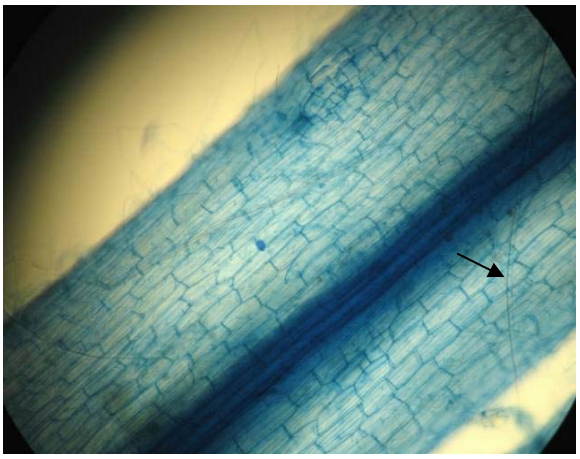


Figure 5. Coiled hyphae of *Trichoderma longibrachiatum*. Cotton blue in lactophenol staining.



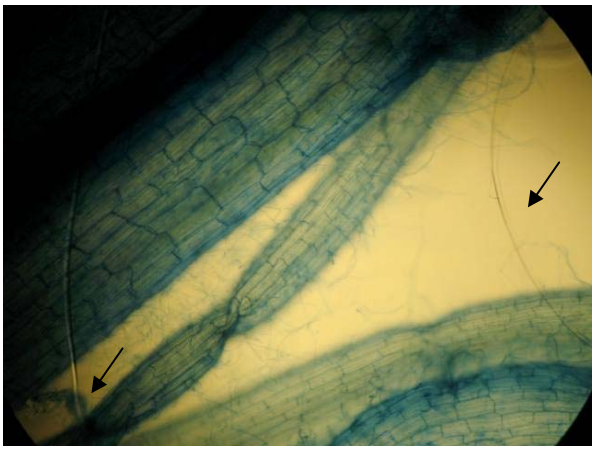


Figure 6. External hyphae of *Trichoderma virens* crossing and coiling the root. Cotton blue in lactophenol

Figure 7. External hyphae of *Trichoderma spp.* surrounding the root. Fresh prep from a young root. No staining.

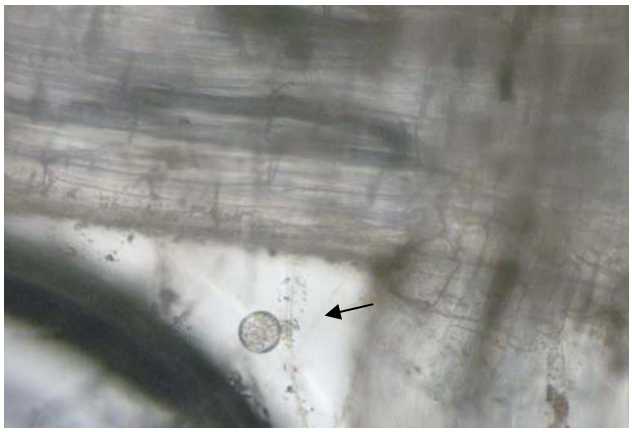


Figure 8. Ungerminated spores of *Trichoderma spp*. No staining.

Figure 9. Infected (right) and non-infected (left) potato plants.

Table 1. Growth parameters of potato plants, after 3 weeks from infection (average between 10 replicates)

Nr. Crt.	Fungi used for infection	Total shoot length (mm)	Number of leaves	Longest root (mm)
1.	Tr. longibrachiatum + AMF	155	10	71
2.	Tr. koningii + AMF	132	8	65
3.	Tr. virens + AMF	135	8	62
4.	Control (non inoculated plants)	130	8	59

Concerning the growth parameters, in case of culturing *Verbena*, *Torenia* and *Diascia* plants propagated from cuttings in association with arbuscular mycorrhizal fungi and *Trichoderma harzianum*, Sramek et al., [6] noticed that the last one had no effect on any

growth parameter nor leaf color; plants in the treatments inoculated with both microorganisms showed similar significantly positive growth response as in the treatments inoculated with mycorrhizal fungi. These reinforce our suspicions that these quantitative results may vary tremendously with the species of plant and of *Trichoderma* used.

Furthermore, the mycoparasitic effect of *Trichoderma* over the mycorrhizal fungi cannot be excluded, as Rousseau *et al.* [7] reported this interaction is possible among other strains. But, while was studied the interaction between the 2 fungi in the presence of a host plant, the above mentioned work used an axenic system. TEM observations of samples from the interaction region showed that hyphae of *T. harzianum* proliferated abundantly at the spore surface and penetrated the thick host wall through local hydrolysis of the wall polymers. This massive colonization was associated with marked cell damage, involving partial to complete disorganization of the cytoplasm, which led in most cases to loss of the protoplasm and apparent bursting of the main hyphae of *G. intraradices*, resulting in the release of the actively proliferating *Trichoderma* hyphae; at an advanced stage of the colonization process, the main hyphae of *G. intraradices* were perforated in many places. These results are not contradictory at all, since the presence of a common host may mediate the aggressiveness of the saprophytic fungus *Trichoderma*. For the spores placed far away from the roots, this parasitic behavior is possible, yet our observation may not have been extensive enough to astound it.

Since the intimate mechanisms of plant response to symbiotic fungi are poorly known, the reason for sharing the physical space in the root must be investigated. Studying this reaction in terms of molecular expression is a more suitable tool, if considering some defense molecules to control the fungi invasion.

Conclusions

Among the 3 strains of *Trichoderma* used, *Tr. longibrachiatum* showed most obvious effect in terms of growth parameters, as well as root length showing signs of infection. All *Trichoderma* species kept expressed their coiling infection pattern.

No root area simultaneously infected with both fungi was observed. No mycoparasitic interaction was found.

It was showed that a mixed fertilizer containing both *Trichoderma* and mycorrhizal fungi would improve plant development and maybe the defense response, thus improving the defense reaction to pathogens. At least for potato, the most indicated *Trichoderma* strain for such a fertilizer is *Tr. longibrachiatum*. One limitation of such a fertilizer may be the compatibility of both fungi category with the host plant, but some very important crop plants like potato and tomato, are still to be considered.

Acknowledgements: This work was supported by a CNCSIS Grant for young researchers (TD 582 / 2007).

Bibliography

1. ILAN CHET, ADA VITERBO AND YARIV BROTMAN. Plant Biocontrol by Trichoderma spp. 2006 (http://www.weizmann.ac.il/Biological_Chemistry/scientist/Chet/Chet.html)
2. SMITH, S.E., READ, D.J., Mycorrhizal Symbiosis. 2nd ed. Academic Press, London, 1997.
3. PHILLIPS J.M., HAYMAN D.S., Improved procedures for clearing roots and staining parasitic and vesiculararbuscular mycorrhizal fungi for rapid assessment of infection, Trans. Brit. Mycol. Soc. **55**, 158–161, 1970.

4. GRACE, C., STRIBLEY, D.P, A safer procedure for routine staining of vesicular-arbuscular mycorrhizal fungi. Mycological Research. **95(10)**, 1160-1162, 1991.
5. MASADEH, B., VON ALTEN, H., GRUNEWALDT-STOECKER, G., SIKORA, R.A., Biocontrol of root-knot nematodes using the arbuscular mycorrhizal fungus *Glomus intraradices* and the antagonist *Trichoderma viride* in two tomato cultivars differing in their suitability as hosts for the nematodes. Journal of plant diseases and protection, **111(4)**, 322-333, 2004.
6. SRAMEK, F., DUBSKÝ, M. VOSÁTKA, M., Effect of arbuscular mycorrhizal fungi and Trichoderma harzianum on three species of balkony plants. Plant Production, **46**, 127-131, 2000.
7. ROUSSEAU, A., BENHAMOU, N., CHET, I., PICHE, Y., Mycoparasitism of the extramatrical phase of Glomus intraradices by Trichoderma harzianum. Phytopathology, 86(5), 434-443, 1996.