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Impact of *Trichoderma harzianum* on the arbuscular mycorrhizal symbiosis demonstrated in an *in vitro* microcosm

Thèse de doctorat présentée par **De Jaeger Nathalie** en vue de l'obtention du grade de Docteur en Sciences agronomiques et ingénierie biologique

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Louvain-La-Neuve, February 2011

A Grand Papa, je dédie cette thèse,
où que tu sois je sais que tu es avec moi.

Acknowledgement

This thesis hadn’t been carried alone; in order to shift from two keystrokes to this final document, many persons have spent some of their time and shared some of their knowledge to help me and to support me. I would like to take now the opportunity to thank all of them.

First, I would like to thank Prof. Stéphane Declerck, for the opportunity he gave me to accomplish this PhD. Thank you for the guidance, the constructive comments and the interesting discussions.

My special thanks go to my supervisor Ivan de la Providencia. I would like to express my gratitude for your continuous encouragements and your complete investment during the course of my PhD. Many thanks for your enthusiasm and for your confidence in me. Simply thank you for being there.

Acknowledgements

I would like to show my gratitude to the people of Cesamm team, with which I spent very nice moments. I enjoyed the lab atmosphere, their friendship, and their support. Special thanks are addressed to the people involved in the project. Thanks to Ingrid van Aarle, for your support, advices and for always making time for me. Thanks to Hervé Rouhier for his investment in my PhD project and for all field excursions. Thanks to Laora Sacré for her technical assistance and for all precious advices for my future life as a mum. Many thanks go to Vanessa Lonnoy for being a caring friend.

I sincerely thank the co-authors of the articles for revising the publications.

Thanks also to the people of CRA-Libramont and especially to Jean-Louis Rolot and Vincent Cesar for providing the necessary facilities and for carrying out field experiments.

I am very grateful to all those persons that were constantly around me during the last four years. Thanks to my family, particularly to my sister, for their non-stop encouragement and their strong support. Many thanks are also addressed to my friends, for their support and all the moments of relaxation.

Finally, I want to thank the « Direction générale opérationnelle de l'Agriculture, des Ressources naturelles et de l'Environnement » of Service Public de Wallonie, for offering me a grant to accomplish this thesis project.

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List of abbreviations

ALP	Alkaline phosphatase
AM	Arbuscular mycorrhizal (fungi)
ANOVA	Analysis of variance
BAS	Branching absorbing structure
BCA	Biological control agent
β-tub	Beta-tubulin (gene)
CFU	Colony-forming unit
Dac	Days after contact
Cp	Crossing point
DNA	Deoxyribose nucleic acid
EF1-α	Elongation factor 1-alpha (gene)
ERM	Extraradical mycelium
GAPDH	Glyceraldehyde phosphate dehydrogenase (gene)
GINCO	Glomeromycota *in vitro* collection
GST1	Gluthatione-S-transferase 1 (gene)
HAM-P	Half-close arbuscular mycorrhizal–Plant (*in vitro* culture system)
HC	Hyphal compartment
HSD	Honest significant difference
IRM	Intraradical mycelium
Lox	Lipoxygenase (gene)
MAPK	MAP kinase (gene)

MDP	Mycelium donor plant (*in vitro* culture system)
MS	Murashige and Skoog (medium)
MSR	Modified of Strullu-Romand (medium)
MUCL	Mycothèque de l'Université catholique de Louvain
P	Phosphorus
PAL	Phenylalanine ammonia lyase (gene)
PDA	Potato dextrose agar (medium)
PGPM	Plant growth promoting microorganism
PPF	Photosynthetic photon flux
PR1	Pathogenesis Related 1 (gene)
PR2	Pathogenesis Related 2 (gene)
PT3	Phosphate transporter 3 (gene)
QRT-PCR	Quantitative real-time-polymerase chain reaction
RC	Root compartment
RH	Runner hyphae
RNA	Ribonucleic acid
ROC	Root organ culture
RT	Reverse transcription
SEM	Scanning electron microscope
SDH	Succinate dehydrogenase
SDWP	Sterile distilled water-peptone
T	*Trichoderma*
Ubc	Ubiquitin conjugating enzyme-like (gene)

Glossary

Alkaline phosphatase: "AM fungi phosphatases are hydrolase enzymes involve in intracellular P transport and transformation, and fungal nutrition. As the name suggests, alkaline phosphatase are most effective in an alkaline environment and are considered as an important enzymes in metabolic process that lead to P transfer to the host plant in the intraradical mycelium, whereas in the extraradical mycelium it may be involved in P uptake from the rhizosphere" (van Aarle and Olsson, 2008).

Arbuscular mycorrhizas: "Widespread type of endomycorrhizal interactions involving fungi of the phylum Glomeromycota, the hyphae of which reach the root inner cortex and develop highly branched exchange structures called arbuscules" (Bonfante and Genre, 2010).

Arbuscule: "Highly branched structure produced by arbuscular mycorrhizal fungi inside the cell lumen of their host. Arbuscules are considered to be the key element of the symbiotic nutrient exchanges between the plant and the fungus" (Bonfante and Genre, 2010).

Biological control agents: "Microorganisms that control disease (primary effect) and have demonstrated stimulation of plant growth (secondary effect) in the absence of a pathogen" (Avis *et al.*, 2008).

Biotrophic (balanced) parasitism: "Relationship where the living host support the growth of the parasite for an extended period of time and may not appear overtly to be little affected, at least in the early stages of the interaction" (Jeffries, 1995)

Coenocytic: "Organism with hyphae lacking septa" (Kirk *et al.*, 2001).

Extraradical mycelium: "Hyphal network that develops in the rhizosphere, in which it absorbs inorganic nutrients that are transferred to the host plant through intraradical hyphae" (Bonfante and Genre, 2010).

Hyphopodium: "Specialized hypha of an arbuscular mycorrhizal fungus, often swollen and usually highly branched that is attached to the host plant epidermis and that initiates intraradical colonization. Hyphopodium is nowadays a synonym of the earlier term appresorium" (Bonfante and Genre, 2010).

Inoculate: "To put a microorganism or a substance containing one, into an organism or a substratum" (Kirk *et al.*, 2001).

Intraradical hyphae: "Network of hyphae from mycorrhizal fungi that colonizes the host root tissues" (Bonfante and Genre, 2010).

Microbial inoculant: "A formulation containing one or more beneficial microorganisms in an easy-to-use and economical carrier

material, either organic, inorganic, or synthesized from defined molecules" (Bashan, 1998).

Mycoparasitism: "Relationship in which one fungus parasitizes another" (Bulter, 1954).

Necrotrophic (destructive) parasitism: "Relationship results in the death and destruction of one or more components of the host mycelium" (Viterbo *et al.*, 2007).

Parasitism: "Relationship in which one microorganism obtains nutrients from another, affecting host viability" (Jeffries, 1995).

Plant growth promoting microorganisms: "Microorganisms that promote plant growth and productivity (primary effect) and have now been shown to also play a role in reducing disease (secondary effect)" (Avis *et al.*, 2008).

Saprotroph or saprobe (saprotrophic adj.): **"**Organism that obtains nutrients from dead organic matter" (Kirk *et al.*, 2001).

Scanning electron microscope: "Type of electron microscope that images the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. The electrons interact with the atoms that make up the sample producing signals that contain information about the sample's surface topography, composition and other properties such as electrical conductivity" (Danilatos, 1988).

Succinate dehydrogenase: "Enzyme complex who bound to the inner mitochondrial membrane of many fungal cells. This enzyme is one of the key enzymes in the Krebs cycle and may be closely related to respiratory activity" (adapted from Saito, 1995).

Sustainable agriculture system: "A system that employ natural processes to achieve acceptable levels of productivity and food quality while minimizing adverse environmental impacts" (Harrier and Watson, 2004)

Transfer by AM fungi: "Successive efflux of ions from the intraradical mycelium of arbuscular mycorrhizas into root cell apoplasm and influx into root cells" (Smith and Read, 2008).

Translocation by AM fungi: "Movement of ions within the hyphae from the extraradical mycelium to the intraradical fungal structures (hyphae, arbuscules and coils) within the roots" (Smith and Read, 2008).

Transport by AM fungi: "Consist of the successive uptake of ions by the extraradical mycelium of arbuscular mycorrhizal fungi, their translocation from the extraradical mycelium to the intraradical mycelium and their transfer from the intraradical mycelium to root cells" (Smith and Read, 2008).

***Trichoderma*: "**Free-living fungi that are know to be opportunistic, avirulent plant symbionts and antagonists for other fungi" (Harman *et al*., 2004).

Uptake by AM fungi: "Uptake of ions by the extraradical mycelium of arbuscular mycorrhizal fungi in the soil" (Smith and Read, 2008).

Summary

The use of beneficial microorganisms has become a reliable alternative to reduce the application of pesticides in modern agriculture. Several studies have demonstrated the beneficial effects of the arbuscular mycorrhizal (AM) fungi and *Trichoderma* spp. to improve plant health and productivity and soil quality. A synergistic effect of both fungi on plant growth as well as on the control of various plant diseases have been shown in numerous studies. However, mycoparasitism by *Trichoderma* spp., a major mechanism to control fungal pathogens, has also been reported on AM fungi. Consequently, for an optimal management of microbial consortia, it is necessary to thoroughly understand the interaction between these two fungi and their respective effects on growth and survival characteristics to guarantee these successful applications under field condition. In this context, the objective of this thesis is to study the interaction of *T. harzianum* (MUCL 29707) with an AM fungi, *Glomus* sp. (MUCL 41833) in order to develop a microbial inoculum containing both beneficial microorganisms. Here we devised *in vitro* microcosm systems to monitor the development of an AM fungus, *Glomus* sp. and *T. harzianum*, via non-destructive microscopic observations. The impact of *T. harzianum* on the AM extra and intraradical mycelium development and functionally (*i.e.* transport of phosphorus) were investigated and the compatibility and applicability of these beneficial microorganisms co-entrapped in alginate beads evaluated. We observed that *T. harzianum* affected the viability of

the extra and intraradical mycelium and the capacity of P transport of the AM fungus when the saprotrophic fungus was inoculated on AM established networks. The saprotroph was able to colonize the root system via a privilege entrance through the hyphae. Interestingly, when *T. harzianum* and *Glomus* sp. were inoculated at the same time, via co-entrapment in alginate beads, this mycoparasitism was infrequently observed, while a stimulation of spore production was noted. This supported the potential of the co-entrapment of both fungi for agro-environmental application. Further questions needs to be addressed regarding the role of AM fungi as a pathway for the entrance of *T. harzianum* into the roots of potato and the ecological significance of co-entrapment of both microorganisms under field condition.

Outlines of the thesis

Based on the current state of knowledge of the interaction between AM fungi and *T. hazianum*, different experiments were conducted to evaluate to possibility of an inoculum formulation containing both fungi (Fig. 4).

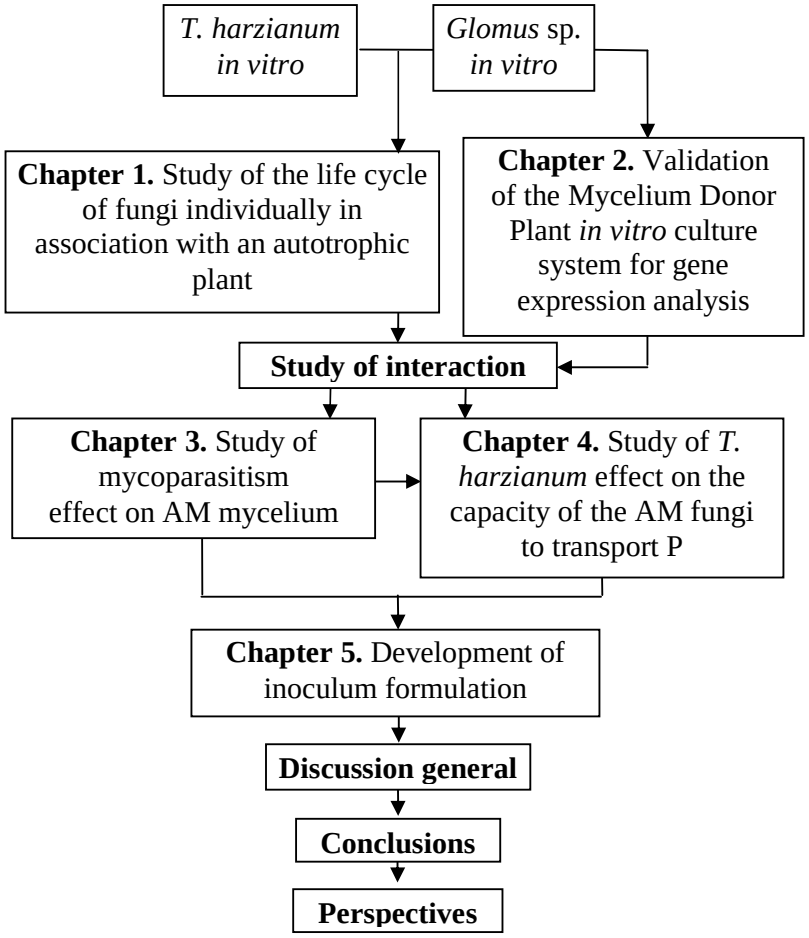

Figure 4. Outline of the thesis.

Outlines of the thesis

After the **Introduction** chapter which presents the subject and objectives of this study, the section **Context of the study** describes the scientific context in which the research was conducted. It contains a brief overview of the literature on the *Trichoderma*-AM fungi interaction topic, detailing what has been discovered in the last decades and describing how researches used the knowledge to manage combination of soil microorganism in modern agriculture.

As the soil is such a complex and interaction-rich environment, interaction studies between microorganisms should be first performed under strictly controlled conditions, so that potentially misleading abiotic and biotic factors could be monitored. The *in vitro* cultivation of AM fungi associated with a photosynthetic plant (named HAM-P) has widely proven its suitability for the study of many aspect of the AM symbiosis. However, this culture system has never been applied to the growth and development of other microorganisms. In **Chapter 1**, it was the objective to adapt and use this *in vitro* culture system to describe and to compare the life cycle of three major fungi, *Glomus* sp., *T. harzianum* and *Rhizoctonia solani* (a potato root pathogen), on autotrophic potato plantlets as a single host.

This work was published in “Spanish Journal of Agricultural Research” (2010).

With the HAM-P *in vitro* culture system described in Chapter 1, the establishment of the AM symbiosis and development of extraradical mycelium network took several weeks. To evaluate the impact of one microorganism on the AM symbiosis development and functionality, a system allowing the fast mycorrhization of seedlings within a few days and rapid re-growth of AM mycelium is thus highly desirable. In **Chapter 2**, we demonstrated the potential of the mycelium donor plant *in vitro* culture system (Voets *et al.*, 2009) as a fast mycorrhization protocol for potato plantlet roots at the seedling stage. During the colonization process, gene expression analysis was performed at different stage of the AM fungal establishment.

This work was published in "Mycorrhiza" (2010).

These two *in vitro* culture systems were used to study the interaction between the saprotrophic, *T. harzianum* and *Glomus* sp. In **Chapter 3**, we investigated the impact of *T. harzianum* on the AM extra and intraradical mycelium development.

This work was published in "FEMS Microbiology Ecology" (2010).

The second interaction experiment (**Chapter 4)**, was focused on the impact of *T. harzianum* parasitism on the physiology (*i.e.* transport of P) of the AM fungi. We studied the uptake, transfer and translocation of ^{33}P by the AM fungi to the host plant in presence of *T.*

harzianum and the impact of *T. harzianum* on the phosphatases activity in the AM extraradical mycelium.

This work was submitted to "FEMS Microbiology Ecology".

Chapter III and IV focused on the symbiotic phase of the AM fungi. Nevertheless, to fulfill the aim of this research project, *i.e.* the development of an inoculum formulation containing the AM fungi and *T. harzianum*, it was necessary to evaluate the impact of *T. harzianum* during the different steps of the life cycle of the AM fungi. Thus, In **Chapter 5**, we investigated the impact of *T. harzianum* on the AM fungal life cycle when both microorganisms are co-entrapped in alginate beads.

This work was submitted to "Journal of Applied Microbiology".

The relevance of this thesis is summarized in the **General Discussion**. In the **Conclusion** section, the major outcomes of this research are given and further commented.

The last section contains the **Perspectives** of this research project, which emphasizes the future improvements for the application of beneficial fungi as viable or complementary alternative of chemicals in modern agriculture.

Author's contribution

The section Introduction and Context of the study at the beginning of this document, and the General Discussion, Conclusions and Perspectives at the end of the thesis have not been published. They were written by myself.

My involvement in **Chapter 1** was the collection and analyzes of the data. I also participated in the redaction and revision of the manuscript. As such, it was estimated that 50% of the work was performed by myself.

My contribution to the **Chapter 2** was 35%. I participated in the design of the experimental procedures, harvest and data collection (*i.e.* characterisation of mycelium donor plant (MDP) *in vitro* culture system, estimation of the intraradical root colonization and data analysis with SAS Enterprise guide). I participated in the revision of the manuscript.

Chapter 3 was fully designed and conducted by myself; co-authors of the chapter have been involved in revising the manuscript. My contribution to this chapter was estimated at 90%.

The work of **Chapter 4** was carried out by myself from setting the goals to publication. My contribution to this chapter was around 90%. Co-authors of the chapter have been involved in revising the manuscript.

Author's contribution

In **Chapter 5**, the design of the experimental procedures, the practical work and data analysis were performed by H. Rouhier and myself. Co-authors of the chapter have been involved in revising the manuscript. My contribution to this chapter was estimated at 80%.

I. INTRODUCTION

Since half a century of intensive agricultural practices (*i.e.* mechanization, plant breeding, pesticides and fertilizers use) to sustain the increased world population food demand (FAO, 2009), the stocks of natural resources (*e.g.* soil, fresh water, biodiversity) have been under constant and increasing pressure (Pimentel and Wilson, 2005). Nowadays, there is a rising interest in the sustainable management of these resources. Among these, the use of beneficial microorganisms has become a viable alternative in agriculture to decrease the detrimental side-effects of, *e.g.* pesticides application and over-fertilization, on human health and environment (Brimmer and Boland, 2003; FAO, 2009; Avis *et al*., 2008).

As stated by van der Heijden *et al.* (2008), "*soil microbes represent the unseen majority in soil"* influencing indirectly and directly the productivity, diversity and composition of plant communities. Over the past 30 years, soil microbes have been described, characterized, and tested for their use as soil quality manager (Schloter *et al*., 2003; Rillig and Mummey, 2006), plant growth promoters and biocontrol agents (Whipps, 2004; Harman *et al*., 2004; Avis *et al*., 2008).

Plant growth promoting microorganisms (PGPMs, *e.g.* Rhizobia, arbuscular mycorrhizal (AM) fungi) can improve plant growth and productivity and are also reported to play a role in reducing disease pressure (Avis *et al*., 2008). Among the PGPM, AM fungi have probably been the most extensively studied. These soil

microorganisms are obligate symbionts that colonize the roots of nearly 80% of terrestrial plants (Smith and Read, 2008). In exchange for carbohydrates, these organisms supply the plants with mineral nutrients and water (Parniske, 2008). Conversely, biological control agents (BCAs, *e.g. Trichoderma* spp., *Pseudomonas* spp.) can control disease and are also reported to stimulate plant growth. *Trichoderma* species are the most extensively studied BCA. These saprotrophic fungi are known to be opportunistic, avirulent plant symbionts and are used as antagonists of other fungi (Harman *et al*., 2004). The *Trichoderma* modes of action are multi-fold including parasitism, competition, antibiosis, and induced plant resistance (Harman *et al.*, 2004). These benefits make the utilization of PGPM and BCA attractive for integrated agricultural practices in view to minimize the impact of chemicals fertilizer and pesticides, or even, in the case of organic agriculture, to eliminate those chemicals from the production system.

The independent use of AM fungi and *Trichoderma* spp. to improve plant growth and to control root pathogens has been largely reported (Harman *et al.*, 2004; Whipps, 2004). The synergetic utilization of both beneficial fungi, exhibiting different or complementary mechanisms of action or abilities to colonize plant roots, appears thus as a promising alternative to reduce the use of fertilizers and to control plant pathogens. Nevertheless, the mechanisms by which *Trichoderma* spp. control pathogens (*e.g.* mycoparasitism) might possibly also impact AM fungi (Calvet *et al.*,

1993; Rousseau *et al.*, 1996; Green *et al.*, 1999; Brimmer and Boland, 2003; Martinez *et al.*, 2004), therefore impeding their ability to coexist into mixed inoculants. Consequently, for an optimal management of microbial consortia, it is necessary to thoroughly understand the interaction between these two microorganisms and their respective effects on growth and survival characteristics (Higa, 1991).

The soil is a complex and interaction-rich environment. Thus interaction studies between microorganisms should be first performed in microcosm controlled systems (*i.e. in vitro* culture systems), so that potentially misleading abiotic and biotic factors can be controlled. Since the initial attempts to culture AM fungi under controlled conditions, many research projects have been carried out to study the effects of *Trichoderma* spp. on the presymbiotic phase of the AM fungi (Calvet *et al.*, 1989; Filion *et al.*, 1999; Martinez *et al.*, 2004). However, few studies have addressed the potential antagonism of *Trichoderma* spp. on the symbiotic phase of the AM fungi (Rousseau *et al.*, 1996). Recently, the *in vitro* culture system associating AM fungi with whole plants (Voets *et al.*, 2005) has paved the way to perform non-destructive microscope observations and to investigate the interaction between microorganisms at organism, cellular and molecular level (Gallou *et al.*, 2009). Here this system is used to approach the intimate coexistence of an AM fungi with *T. harzianum* to better understand their mode of (inter)action.

The objective of this thesis is to study the interaction of *T. harzianum* (MUCL 29707) with an AM fungus, *Glomus* sp. (MUCL 41833) in order to develop a microbial inoculum containing both beneficial microorganisms. In details, it is the objective to improve our understandings on the effects of *T. harzianum* on the AM fungal morphology (*i.e.* extra- and intraradical development) and physiology (*i.e.* transport of P). Using the autotrophic *in vitro* culture system (Voets *et al*., 2005, 2009), the direct microscopic non-disturbed observation of interaction between *T. harzianum* and *Glomus* sp. were investigated. To evaluate the impact of *T. harzianum* on the symbiosis functionality, this system was aslo used to perform enzymatic analysis and transport study. The significance of parasitism of the AM fungi was discussed.

II. CONTEXT OF THE STUDY

1. Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal (AM) fungi are obligate symbionts in the phylum Glomeromycota (Schuβler *et al.*, 2001). It establish a mutualistic relationship which the roots of terrestrial plant (Smith and Read, 2008). The AM fungi supply the plants with mineral nutrients (*i.e.* P, N, K, Cu, Zn) and increase their water uptake in exchange for carbohydrates (Smith and Read, 2008). The AM symbiosis is considered 'non-specific' and as a consequence, it is estimated that the AM fungi live in association with the roots of nearly 80% of land plants (Smith and Read, 2008). The observation of Remy *et al.* (1994) in fossils of Rhynia suggested that the origins of the AM fungi date back 400 million years ago. Thus it seems likely that this symbiont could have assisted vascular plant in the colonization of land (Simon *et al.*, 1993).

Arbuscular mycorrhizal fungi develop within two niches: within the cells of the root, called the intraradical mycelium (IRM) and in the surrounding soil, named the extraradical mycelium (ERM) (Fig. 2). The coenocytic nature (*i.e.* the absence of septa in living hyphae) of the AM mycelium linked to the protoplasmic continuity facilitates the transport of resources from source to sink regions of the fungal colony and/or symbiotic root (Smith and Read, 2008). The role of the ERM consists in the uptake and translocation of nutrient and water to the plant roots from locations beyond the rhizospheric zone (Jakobsen *et al.*, 1992; Johansen *et al.*, 1993). In addition, the ERM

contributes largely to soil quality (*i.e.* soil aggregation) (Schloter *et al.*, 2003; Rillig and Mummey, 2006). The IRM is involved in the transfer of nutrients from the AM fungus to the plant. The bidirectional exchange occurs at the level of tree-shaped subcellular structures within root cells, the arbuscules (Fig. 2a-c). These structures are thought to be the main site of nutrient exchange between the fungal and plant symbiotic partners.

The AM symbiosis confers a range of additional benefits for the plant. They increase the plant tolerance to environmental stresses (*i.e.* drought, salinity), induce greater resistance to pathogens and reduce sensitivity to toxic substances present in the soil (Azcon-Aguilar and Barea, 1996; Avis *et al.*, 2008).

1.1. Phylogeny of AM fungi

AM fungi are a monophyletic group of soil fungi, reclassified from the polyphyletic phylum *Zygomycota* to a new phylum *Glomeromycota* (Schuβler *et al.*, 2001). Inside of the phylum, according to SSU sequence phylogeny, four orders encompassing eleven families and seventeen genera have been defined recently (Table 1). (http://www.lrz.de/~schuessler/amphylo/, updated December 2010, consulted 25th January 2011). The largest "genus" within the AM fungi, *Glomus*, is not monophyletic, with members distributed in at least four families: *Glomus* groups A, B, *Diversisporaceae* (Schwarzott *et al.*, 2001).

Table 1 Orders, families, genera in Glomeromycota and species distribution per genus.

Order	Family	Genus	Number of described species[1]
Glomerales	*Glomeraceae*	*Glomus*	78
		Funneliformis	11
		Rhizophagus	9
		Sclerocystis	10
	Claroideoglomeraceae	*Claroideoglomus*	6
Diversisporales	*Acaulosporaceae*	*Acaulospora*	37
	Entrophosporaceae	*Entrophospora*	3
	Diversisporaceae	*Diversisporaa*	6
		Otospora	1
	Gigasporaceae	*Gigaspora*	8
		Scutellospora	28
		Racocetra	11
	Pacisporaceae	*Pacispora*	7
Archaeosporales	*Archeosporaceae*	*Archaeospora*	2
	Ambisporaceae	*Ambispora*	9
	Geosiphonaceae	*Geosiphon*	1
Paraglomerales	*Paraglomeraceae*	*Paraglomus*	3
Total 4	11	17	230

[1] The total number of species includes synonymous species (updated December 2010, consulted 25th January 2011).

1.2. AM fungal life cycle

Present evidences indicate that AM fungi only reproduce asexually. So far no report substantiates a possible sexual reproduction. Besides, AM fungi are coenocytic microorganisms (*i.e.* without septa in their hyphae) and their mycelium produces multinucleated spores. Therefore these fungi do not have any monokaryotic life stage (Bever *et al.*, 2008; Parniske, 2008).

Within soil, AM fungi reproduce via spores, infected root fragments and hyphae, collectively termed propagules. However, the capacity of these propagules to colonize roots seems to differ between genera. Klironomos and Hart (2002) demonstrated that *Glomus* and A*caulospora* were able to colonize roots from all three inoculum sources, while S*cutellospora* and *Gigaspora* appear to depend mostly entirely on spores.

The life cycle of AM fungi is separated in three phases: asymbiotic, pre-symbiotic and symbiotic (Fig. 1) (Bucher *et al.*, 2009).

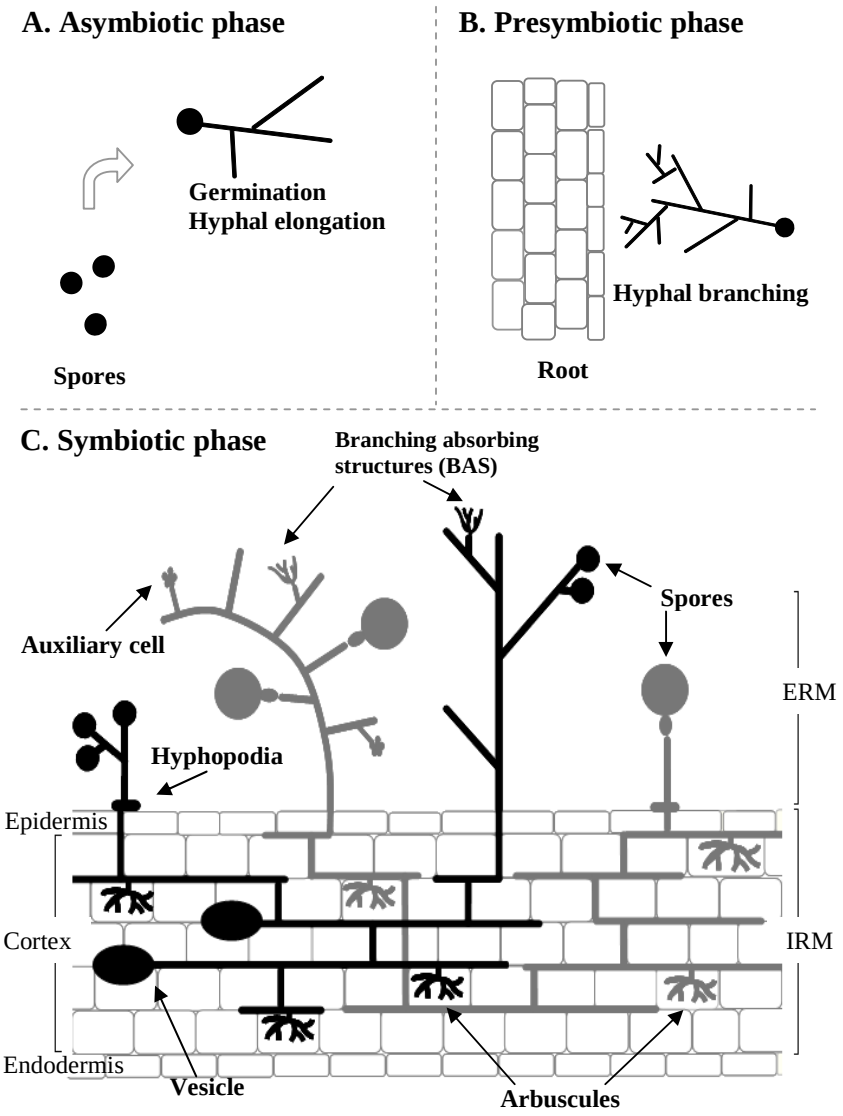

Figure 1 Life cycle of arbuscular mycorrhizal fungi. A. Asymbiotic phase. B. Presymbiotic phase. C. Symbiotic phase. Extraradical and intraradical development of AM fungus of the *Glomeraceae* (black) and *Gigasporaceae* (gray) family and, developing Arum-type colonization. Adapted from Akiyama (2007).

a. Asymbiotic phase

The asymbiotic phase is characterized by the absence of signals from the host plant that may trigger germination or hyphal growth (Breuninger and Requena, 2004). This phase is associated with the production of a limited amount of branched, coenocytic hyphae, which are capable of anastomosis. Even though most spores in the soil are capable to germinate in the absence of a suitable host root, the germination process is improved by root extracts (*e.g.* lipophilic compounds identified as a sesquiterpene (Akiyama *et al.*, 2005; Besserer *et al.*, 2006; Smith and Read, 2008) or microbial exudates (Calvet *et al.*, 1992; Fracchia *et al.*, 2004; Martinez *et al.*, 2004). However, if no root colonization occurs, the hyphae ceases growth after a few days and cytoplasm, nuclei and cellular organelles retracts following sequential septa formation in hyphae (Giovannetti, 2002). In the vicinity of a host plant, root signals elicit the AM fungi to switch from the asymbiotic to the presymbiotic phase (Tamasloukht *et al.*, 2003; Buée *et al.*, 2000).

b. Presymbiotic phase

The pre-symbiotic phase comprises the host recognition prior to physical contact (Requena *et al.*, 2007; Bresserer *et al.*, 2006). The host recognition is mediated by a complex signaling process between the plant and the AM fungi (Bucher, 2007; Requena *et al.*, 2007; Bonfante and Genre, 2008) characterized by physiological changes in

both partners (Gutjahr *et al.*, 2009; Akiyama *et al.*, 2005). These changes have the function of stimulating growth and branching of mycelium and differencing hyphal structure to form a hyphopodium, via which it enters the root (Genre *et al.*, 2008). A this stage the symbiotic phase is initiated.

c. Symbiotic phase

The symbiotic phase is initiated with the development of penetrating hyphae (hyphopodium), and in most cases the hyphal extensions in the epidermal and outer cortical cells (Bonfante and Genre, 2008). The pattern of mycelium growth within the root is depending on the plant species involved. Gallaud (1905) described two general anatomical groups: the *Arum*-type (Fig. 2a, b) and *Paris*-type (Fig. 2c). In the *Arum*-type, hyphae spread longitudinally to the cortical cells. Short side branches penetrate the cortical cell walls and branch dichotomously in the cell lumen to produce highly branched arbuscules, forming an interface between the fungal tissues and the plant plasma membrane (Fig. 2a, b). In the *Paris*-type, cortical colonization of roots is characterized by extensive development of intracellular coiled hyphae and arbusculate coils (*i.e.* intercalary arbuscules) which spread directly from cell to cell (Fig 2c). Identically, these structures are believed to be the preferential site for the bi-directional nutrient exchange with the plant (Bonfante-Fasolo, 1984; Rausch *et al.*, 2001). However, it appears that the arbuscules are not the only intraradical structures supporting the bidirectional C-

mineral flow between the two partners of the symbiosis and those intraradical hyphae are also involved in such exchange (Gianinazzi-Pearson *et al.*, 1991; van Aarle *et al.*, 2005; Schüßler *et al.*, 2006). These two colonization patterns were recently revisited in the so-called '*Arum-Paris* continuum' model, which describes the possible range of intermediate patterns in relation with the identity of the fungal and plant partners (Bonfante and Genre, 2008).

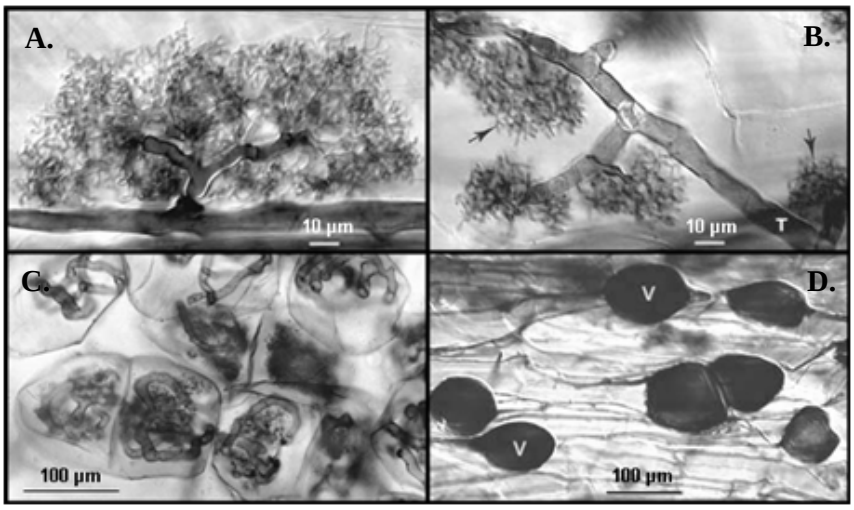

Figure 2 A. Mature arbuscule of *Glomus mosseae* with numerous fine branch hyphae. From Brundrett M. B. Arbuscule of *Gigaspora margarita* with an elongated trunk hypha (T) and tufts of fine branch hyphae (arrows) From Brundrett M. Note how this arbuscule differs from the *Glomus* arbuscules above. C. Arbuscular mycorrhizal development in American ginseng *(Panax americana),* showing highly developed coils in cortical cells, occasionally subtending sparse arbuscules. Note absence of intercellular hyphae; colonization spreads directly from cell to cell. From Peterson *et al*. (2004) D. Vesicles (V) produced by a *Glomus* species in a leek root. This root also contains many intercellular hyphae From Brundrett M.

In parallel to arbuscules, AM fungi belonging to the *Glomeraceae*, *Acaulosporaceae*, *Entrophosporaceae* and *Gerdemanniaceae* develop inter- or intracellular vesicles within the roots, serving as storage organs and as reproductive structures (Fig. 2d) (Declerck *et al.*, 1998; Smith and Read, 2008). Such structures are never observed in the *Gigasporaceae*.

Once symbiosis with a suitable host has been established, fungal hyphae exit from roots spreading into the surrounding soil, where it develops as a dense ERM network. Classical description of the ERM reported a dimorphic hyphal nature (Friese and Allen, 1991), with coarse straight-growing thick-walled hyphae, greater than 20 µm diameter (*e.g.* runner hyphae (RH)) and fine, thin-walled hyphae of 2-10 µm (Mosse, 1959). The architecture of this network may differ between *Glomeracea* and *Gigasporaceae* species (Bago *et al.*, 1998a, b; de Souza and Declerck, 2003) (Fig. 3).

In *Glomus* sp., the ERM spread radially in the substrate and is lead by thick RH which form the skeleton of the fungal colony (Bago *et al.*, 1998a) (Fig. 3d). These thick branched at angles about 45° to form branched hyphae (BH) providing higher order hyphae, often interconnected by means of hyphal fusions (*i.e.* anastomoses) to form a three-dimensional hyphal network (Bago *et al.*, 1998a). In the *Glomeraceae* anastomosis were observed between different hyphae of the same or independent network (de la Providencia *et al.*, 2005; Voets *et al.*, 2006). Hyphae form characteristic structures including

branched absorbing structures (BAS) described as preferential structures for nutrients uptake (Fig. 3f) (Bago *et al.*, 1998b).

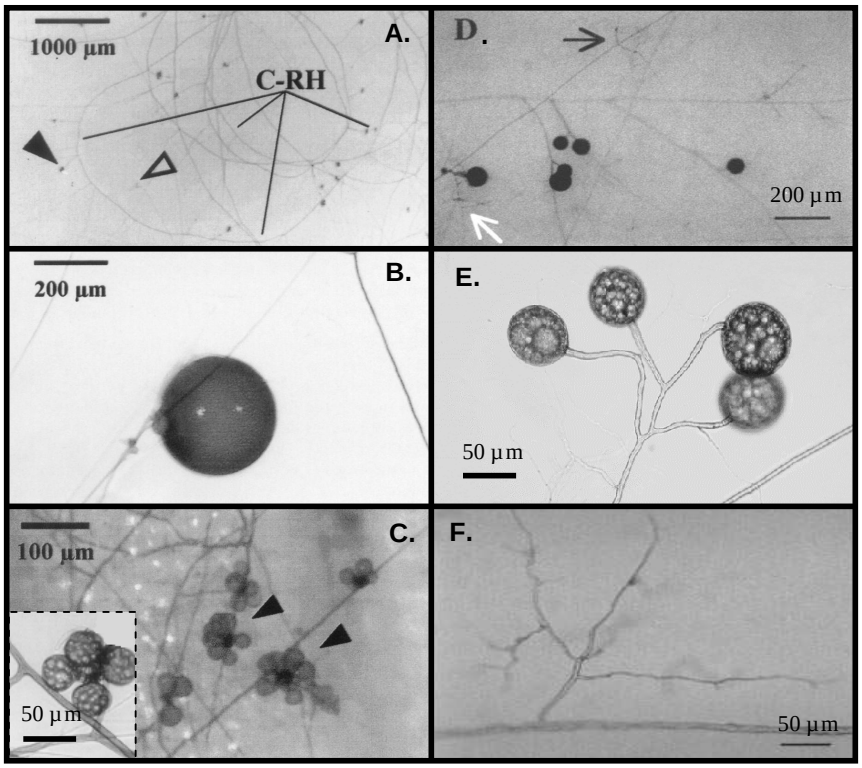

Figure 3 A-C. Mycelium architecture of *Scutellospora reticulata* growing in Ri T-DNA transformed carrot root. From de Souza and Declerck (2003). A. General view of the mycelium architecture showing circular-runner hyphae (C-RH) and secondary hyphae bearing numerous auxiliary cells (bold arrowhead). Hollow arrowhead shows sporogenous hypha bearing an immaturespore. B. S. reticulata mature spore. C. Auxiliary cells in the vicinity and on a root surface. Inset: Mature auxiliary cells with smooth surface and filled with lipid droplets. D-F Mycelium architecture of *Glomus* sp.growing in Ri T-DNA transformed carrot root. D. Extraradical mycelium showing branched absorbing structures (BAS)(black arrow) and spore-associated BAS (white arrow). From Bago *et al*., (1998a). E. *Glomus* sp. mature spores. From GINCO (http://www.mycorrhiza.be/ginco-bel/index.php). F. Dichotomously branched short ramication (branched absorbing structure, BAS) developing on a runner hypha of *Glomus* sp. in a monoxenic symbiotic culture. From Bago *et al.* (1998b).

In *S. reticulata* (a *Gigasporaceae*) two major architecture patterns are observed; RH formed as open spirals or only slightly curved (Fig. 3a) (de Souza and Declerck, 2003). The anastomosis mechanism was seldom observed and anastomoses were essentially formed by hyphal bridges within the same hyphae (de la Providencia *et al.*, 2005). These authors also described the existence of BAS-like structure in *Gigasporaceae* mycelium, but these structures were less ramified than those described by Bago *et al.* (1998b).

After extensive colonization by RH and BAS development, storage organs are formed on the external mycelium. New spores develop terminally or intercalary on hyphae (de Souza and Berbara, 1999). Each spore contains a huge number of haploid nuclei, storage lipid and carbohydrates (Smith and Read, 2008). In the *Glomeraceae*, the spores are globose to subglobose (30-120 µm diam), pale yellow to greyish yellow (Fig. 3e). Spores of *Gigasporaceae* develop blastically from a bulbous sporogenous cell formed at the tip of hyphae (Fig. 3b). Spores are yellowish white to black; globose to subglobose; (260-405 µm diam). *Gigasporaceae* AM extraradical mycelium is characterized by the formation of auxiliary cells (Fig. 3c) (Morton and Benny, 1990). These structures are rich in nuclei, organelles and lipids (Declerck *et al.*, 2004) and have been suggested as carbon storage and reproductive structures (de Souza and Declerck, 2003).

1.3. AM fungi and plant disease control

AM fungi are often reported as plant growth promoting microorganisms (PGPM) (Avis *et al.*, 2008). In addition, their involvement in the control of plant pathogens has also been mentioned repeatedly in the last decades, supported by a wealth of recent reviews (Azcón-Aguilar and Barea 1996; Whipps, 2004; Avis *et al.*, 2008).

A number of studies reported reductions in incidence and/or severity of root rot or wilting caused by fungi such as *Rhizoctonia*, *Fusarium* or *Verticillium*, and root rot caused by Oomycetes including *Phytophthora*, *Pythium* and *Aphanomyces.* Different mechanisms have been postulated such as (i) improved nutritional status of the plant (Norman *et al.* 1996; Smith and Read, 2008); (ii) direct competition for carbohydrates (Azcon-Aguilar and Barea, 1996) and space within the mycorrhizosphere and the host roots (Cordier *et al.*, 1996, 1998); (iii) direct and indirect interaction within the rhizosphere by hyphal exudation and modulation of root exudation inducing changes in composition and activity of microbial communities (Andrade *et al.* 1997; Citernesi *et al.* 1996; Barea *et al.*, 2005) and (iv) biochemical changes associated with plant defence mechanisms and induced resistance (Pozo *et al.*, 2010) (Fig. 4).

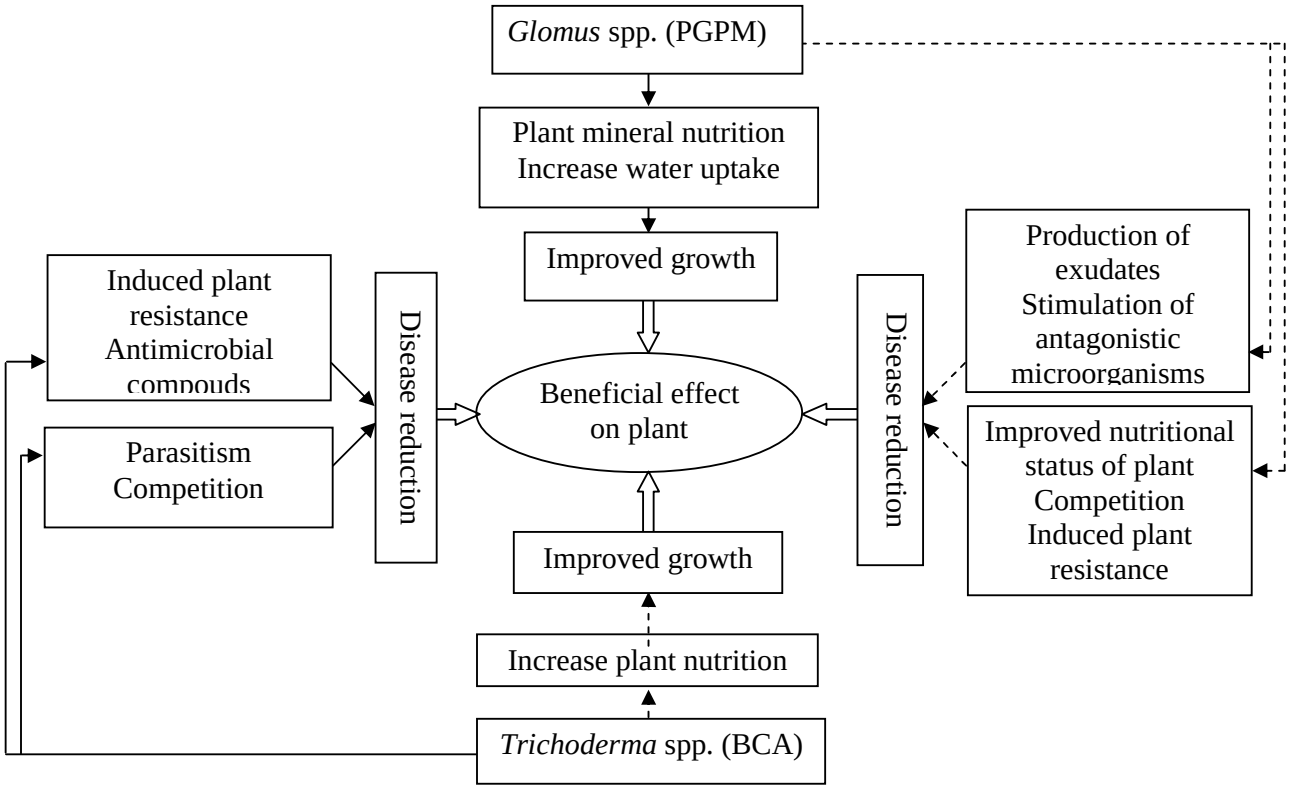

Figure 4 Potential modes of action of plant growth promoting microorganism (PGPM) and biological control agent (BCA) with primary and secondary beneficial effects on plant. Solid lines, primary effect; dash lines, secondary effect (Modified from Avis *et al.*, 2008).

According to the results obtained in recent studies, the establishment of the AM symbiosis induces important changes in the physiology of the host plant including activation of specific local and systemic plant defence mechanisms (Pozo *et al.*, 2010). The increased lignification of root endodermal cells induced by AM colonization has been suggested to play an important role in the plant defence mechanism (Dehne, 1982). The accumulation of phytoalexins, enzymes of the phenylpropanoid pathway, chitinases, β-1,3-glucanases, peroxidases, pathogenesis-related (PR) proteins, callose, and phenolics (Gianinazzi-Pearson *et al.*, 1994; Cordier *et al.*, 1998; Pozo *et al.*, 1996, 1998, 1999) were increased in mycorrhizal plants. These plant responses are generally localized.

Several studies have demonstrated that AM fungi may predispose the whole plant to an early response to attack by a pathogen (Gianinazzi-Pearson *et al.*, 1994; Pozo and Azcón-Aguilar, 2007). This boost of basal defences is known as *priming* (Conrath *et al.* 2006). Evidence is accumulating that priming is associated to systemic resistance or mycorrhiza-induced resistance (MIR) (Cordier *et al.* 1998; Pozo *et al.* 2009) and are regulated by jasmonate signalling pathways (reviewed in Pozo *et al.*, 2010). This mechanism contrast with the systemic acquired resistance (SAR) triggered in plants after infection with necrotising pathogens (Pozo and Azcón-Aguilar, 2007). Typically, the SAR relies on salicylic acid produced by the plant as a signalling molecule. Nevertheless, the jasmonate and salicylate induced pathways are characterized by the production of a

cascade of PR proteins (reviewed in Conrath *et al.*, 2006). These include antifungal chitinases, glucanases and thaumatins, and oxidative enzymes, such as peroxidases, polyphenol oxidases and lipoxygenases. Low-molecular-weight compounds with antimicrobial properties (phytoalexins) can also accumulate in tissues (Conrath *et al.*, 2006).

Different mechanisms have been reported to play a role in plant protection by AM fungi. Implicit in all the mode of action studies is that more than one mechanism can be involved in any single AM fungi/plant/pathogen combination. Several mechanisms can be operative simultaneously, their respective contributions depending on the environmental conditions, timing of the interaction and partners involved (Azcón-Aguilar and Barea 1996; Whipps, 2004).

1.4. AM fungi in agriculture

The roles of AM fungi in soil aggregation and stabilization, in improved plant disease tolerance and in increased mobilization of nutrients represent attractive properties for biological farming and sustainable management practices in agricultural systems (Smith and Read, 2008). The application of AM fungi are now integrated into a wide variety of growing systems as part of integrated pest and management practices (Atoun and Prévost, 2005; Berg, 2009).

a. Case study: AM fungi and potato crop

Potato (*Solanum tuberosum*) is an herbaceous annual plant ranking as the world's fourth most important food crop (FAO, 2009). In intensive potato crops, the use of herbicides, fertilizers and pesticides are important and there is no conclusive chemical control of bacteria and viruses. Increasing potato production while protecting producers, consumers and the environment requires a holistic crop protection approach that may include the use of beneficial microorganisms like the AM fungi.

The beneficial effect of AM fungi on potato plant has been reported over the last decades (Graham *et al.*, 1976; Yao *et al.*, 2002, 2003, Ryan *et al.*, 2000, 2003). A number of studies have demonstrated that inoculation of AM fungi under field conditions increased plant growth and tuberization of potatoes (Graham *et al.*, 1976, Larkin, 2008). Under nutrient deficient conditions, an increase in mineral nutrition (*i.e.* increased shoot concentrations of P, N and Mg) was reported in mycorrhizal potato plants (Black and Tinker, 1977; Gaur and Adholeya, 2000; McArthur and Knowles, 2003). The same observations were made in a high P agricultural soil (Douds *et al.*, 2007). Several studies have shown that tuber size distribution and tuber yield increased in presence of AM fungi (Douds *et al.*, 2007). AM fungi have also been shown to increase plant resistance to potato fungal root pathogens such as *Rhizoctonia solani* via the accumulation of metabolic compounds in root tissues (*i.e.* phytoalexins) (Yao *et al.*,

2002; 2003). Damage by nematodes and increase of plant tolerance against root-lesion nematodes was also demonstrated (Ryan *et al.*, 2003; Dong and Zhang, 2006). Other results suggested that AM fungi inoculation during minituber production influence the postharvest progression of tuber dry rot caused by *Gibberella pulicaris* (anamorph.=*Fusarium sambucinum*) suggesting a persistent systemic resistance (Niemira *et al.*, 1996).

b. Management of AM fungi in agricultural systems

Crop management used to meet production goals involves a range of practices which can be potentially detrimental for the AM fungal association (Gosling *et al.*, 2005; Rasmann *et al.*, 2009); particularly in terms of diversity (Menendez *et al.*, 2001; Verbruggen and Kiers, 2010).

Intensive agriculture is characterized by high N and P inputs. Under nutrient-rich conditions, host plants may severely reduce or even interrupt resource allocation to their fungal partners, resulting in decreased AM fungal root colonization (Smith and Read, 2008). In this context, Siddiqui and Pichtel (2008) demonstrated that the beneficial effects of AM fungi are most apparent when nutrients are limiting.

The effect of fungicides on the AM association is complex and not easily predicable (Perrin and Plenchette, 1993; Schreiner and Bethlenfalvay, 1997). Several authors demonstrated that the use of

fungicides (*i.e.* mefenoxam, fenpropimorph or fenhexamid) at recommended application rates reduced AM colonization and sporulation (Campagnac *et al.*, 2008, 2009; Zocco *et al.*, 2008), while other studies (*i.e.* fungicides contained carbendazim, fludioxonilor, a mixture of propiconazole and fenpropimorph or a mixture of 1,3-dichloropropene and chloropicrin as their active ingredients) did not report any effect on the symbiosis (Schweiger *et al.*, 2001; Burrows and Ahmed, 2007; Murillo-Williams and Pedersen, 2008; Zocco *et al.*, 2010)

Tillage-mediated soil disturbance has been shown to disturb the network of hyphae in soil, with consequent reductions in root colonization, nutrient acquisition and hence crop yield (Gosling *et al.*, 2005; Smith and Read, 2008).

The inclusion of non-mycorrhizal crops within rotations was associated to a decrease of AM propagules in the soil, decreasing both AM fungal root colonization and yield of subsequent crops (Douds *et al.*, 1997; Arihawa and Karasawa, 2000). In addition to crop succession, varietals selection, cultivation and fallowing have been shown to affect mycorrhizal activity (Hetrick *et al.*, 1996; McGonigle and Miller, 2000).

It was assumed that the agricultural practices mentioned above could affect the mycorrhizal diversity. It was suggested that AM fungi in the *Gigasporaceae* are poorly adapted to soil disturbance (de la

Providencia *et al.*, 2007), thus soils used for agricultural production are often dominated by *Glomus* species (Menendez *et al.*, 2001; Rosendahl and Matzen, 2008; Verbruggen and Kiers, 2010). AM fungi in the *Gigasporaceae* have been shown to functionally complement other AM fungal families (*e.g. Glomeraceae*, *Acaulosporaceae*) in the P nutrition of plants due to their higher soil-hyphal density (Maherali and Klironomos, 2007). Consequently, their elimination from agricultural systems represents a potential loss of useful functional diversity for crop hosts (Verbruggen and Kiers, 2010).

In recent years, particular attention has been paid to the management of natural soil resources. To maintain AM fungal populations in soil used for agriculture at a reasonable level, there are essentially two approaches: (i) the application of management practices which maintain and increase AM fungi indigenous community and (ii) the inoculation (and subsequent management) of selected AM fungi (Smith and Read, 2008).

As rehabilitation of diverse and effective AM fungi community may take a long time (Scullion *et al.*, 1998), the use of commercial inoculum may be the best way to reintroduce AM fungi community (Eason *et al.*, 1999). At present, AM fungal inoculum is produced using different biotechnologies from relatively simple techniques (*i.e. in vivo* production in containers with different substrates) to more complex ones (*i.e. in vitro* methods) (Ijdo *et al.*, 2010) and different

formulations are available on the markets (Gianinazzi and Vosatka, 2004). However, few agricultural systems use AM fungi inoculum because of the high cost of production, the difficulty of application, and uncertainties related to the competitive ability of AM fungi under field situation (Gianinazzi and Vosatka, 2004).

2. *Trichoderma* spp.

Trichoderma spp. is a genus of free-living fungi common in soil and roots (Harman *et al.*, 2004). These fungi have been described as saprotrophic organisms, although some of them are, or have the capacity to be, mycoparasites (Barnett and Binder, 1973). Many species in this genus have been characterized as opportunistic avirulent plant symbionts and function as antagonists of many phytopathogenic fungi, thus protecting plants from disease (Harman *et al.*, 2004).

2.1. Classification of *Trichoderma* spp.

Trichoderma spp. is a genus of asexually reproducing fungi belonging to the *Hypocreaceae* family. Teleomorphs (sexual form of a fungus) of *Trichoderma* are species of the Ascomycete genus *Hypocrea* (Kubicek and Harman, 1998). However, many strains, including most biocontrol strains, have no known sexual stage (Harman *et al.*, 2004). *Trichoderma* spp. have a high level of genetic diversity which makes them easily adaptable to divers environments (Harman *et al.*, 2004).

2.2. *Trichoderma* sp. life cycle

As described by Kubicek and Harman (1998), the organism grows and ramifies as typical fungal hyphae, 5 to 10 µm in diameter. The mycelium is composed of hyaline, septate, much-branched and smooth-walled hyphae. The conidiophores are highly ramified tending

to be regularly verticillate forming a pyramidal structure (Fig. 5a, b). The main branches of conidiophores produce numerous shorter side branches arising in groups of three (Fig. 5a). The apex of each branch is terminated by phialides. Asexual sporulation occurs as single-celled subglobose to obovoid, smooth-walled, usually green, conidia (Fig. 4c) (typically 3 to 5 µm in diameter) that are produced successively and accumulate at the apex of the phialides. Intercalary or occasionally terminal resting chlamydospores are also formed, these also are single celled, globose or ellipsoidal, colorless and smooth-walled. In culture, colonies grow rapidity, at first smooth-surfaced and watery white, later becoming compactly tufted, of various shades of green.

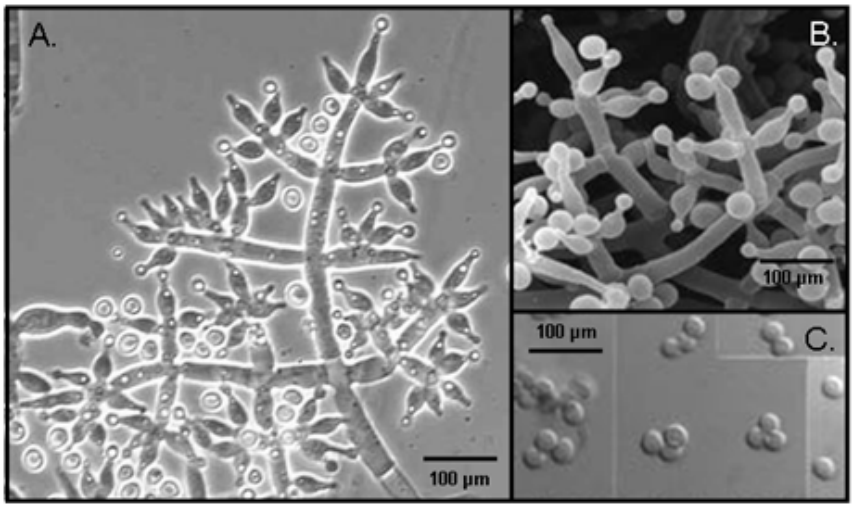

Figure 5 A-C Mycelium architecture of *Trichoderma harzianum*. A Conidiophore and conidia. From Gams W. B. Conidiophores of *T. harzianum*. From Hoog G.S. C. Smooth-walled conidia of *T. harzianum*. From Hoog G.S.

2.3. *Trichoderma* sp. and plant control disease

Trichoderma sp. are among the most studied biocontrol agents (BCA). In addition to the effectiveness of *Trichoderma* sp. in controlling plant pathogens (*i.e. Rhizoctonia solani*, *Fusarium oxysporum*, *Pythium*, *Botrytis cinerea*, etc.), many studies have shown that these BCAs have properties that directly promote plant growth (Harman, 2000; Yedidia *et al.*, 2001; Vinale *et al.*, 2007). Depending upon the strain, the mode of action of *Trichoderma* sp. may include (i) mycoparasitism (Rousseau *et al.*, 1996; Benhamou and Chet, 1997), (ii) competition for nutrients (*i.e.* carbon, nitrogen,…) and space (*i.e.* specific infection sites) (Gullino, 1992; Sivan and Chet, 1989), (iii) production of antibiosis and secondary metabolites (Ghisalberti and Sivasithamparam, 1991) (iv) improved root and plant growth (Harman, 2000; Chacon *et al.*, 2007), and (v) induction of resistance in plants (Yedida *et al.*, 1999) (Fig. 4). Recent reviews (Harman *et al.*, 2004; Vinale *et al.*, 2007; Avis *et al.*, 2008; Rincon *et al.*, 2009) present a compilation of the most recent advances in understanding the mechanisms involved in the interaction of *Trichoderma* sp. with microbes and plants.

2.4. The mycoparasitism

Mycoparasitism describes a relationship in which one microorganism obtains nutrients from another, affecting the host viability (Jeffries, 1995). Barnett and Binder (1973) divided mycoparasitism into (i)

necrotrophic parasitism, in which the relationships result in death of the host; and (ii) biotrophic parasitism, in which the development of the parasite is favored by a living rather than a dead host structure. In *Trichoderma* sp., mycoparasitism has been found to be necrotrophic and rather specific (Viterbo *et al.*, 2007). *Trichoderma* sp. have a wide host range (*e.g. Rhizoctonia solani* (Elad *et al.*, 1983, 1987), *Sclerotium rolfsii* (Elad *et al.*, 1983), *G. intraradices* (Rousseau *et al.*, 1996)).

Mycoparasitism by *Trichoderma* sp. is a sequential process that involves the detection of the fungal host, contact, attachment and host penetration (Harman *et al.*, 2004; Vinale *et al.*, 2007). To recognize potential host, *Trichoderma* sp. secretes cell wall degrading enzymes (CWDEs: cellulases, chitinases, glucanases, etc.) (Lorito, 1998; Harman *et al.*, 2004; Vinale *et al.*, 2007). In the environment, these high molecular weight compounds are diffused and hydrolyze the cell wall of the host fungus (Kubicek *et al.*, 2001; Howell, 2003; Woo *et al.*, 2006). It is believed that *Trichoderma* sp. detects the presence of another fungus by sensing the molecules released from the host by enzymatic degradation (Harman *et al.*, 2004; Lorito *et al.*, 2006; Woo and Lorito, 2007) (Fig. 6). *Trichoderma* sp. grows towards its host by chemotropism along the cell-wall oligomers gradient.

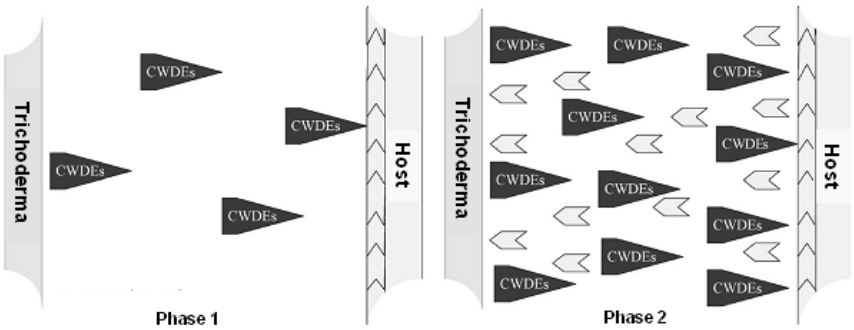

Figure 6 The pre-contact events of the mycoparasitic interaction *Trichoderma*-host fungus. Phase 1: the mycoparasite produces high molecular weight compounds that reach the host. Phase 2: low molecular weight-degradation products that are released from the host cell walls reach the mycoparasite and activate the mycoparasitic gene expression cascade (modified from Vinale *et al*., 2007).

Once the fungus come into contact, *Trichoderma* sp. attach to the host by binding carbohydrates of their cell walls to the lectins of the target fungi (Inbar *et al.*, 1996). *Trichoderma* sp. can coil around the host or attach on the host surface as 'T-shaped branch' structures (Gupta *et al.*, 1999; Harman *et al.*, 2004) and form appressoria (Rousseau *et al.*, 1996; Harman *et al.*, 2004). At the site of the appressoria, *Trichoderma* sp. degrades the host wall by producing CWDEs and probably peptaibol antibiotics (Schimbock *et al.*, 1994). The holes produced allow to *Trichoderma* sp. to penetrate into the lumen of the host and to assimilate the intracellular content (Benhamou and Chet, 1997). *Trichoderma* sp. not only parasitize the hyphae of many fungal species but also can penetrate and destroy some of the resting structures (*i.e.* sclerotia, spores), thereby reducing their inoculum potential in soil (Lee and Koske, 1994).

The mycoparasitism activity is regulated by host induced genes and genes induced upon release of catabolite repression (Viterbo and Horwitz, 2010). The mycoparasite, growing initially as a saprophyte in a nutrient-scarce environment (*i.e.* under depleted carbon or nitrogen sources), may elicit genes encoding CWDEs that will enable it to attack a host whenever it becomes available (Benitez *et al.*, 1998; Lorito, 1998). It was also suggested that mycoparasitism may be mediated by different environmental stress including pH (Moreno-Mateos *et al.*, 2007), and that antagonism itself is a stress situation (Olmedo-Monfil *et al.*, 2002).

3. Interaction of the AM fungi and beneficial saprotrophic *Trichoderma* sp.

In sustainable, low-input cropping systems the roles of microorganisms in maintaining soil fertility, improving plant nutrition and biocontrol of plant pathogens may be more important than in conventional agriculture in which high inputs of agrochemicals are used. Numerous studies have reported that the application of beneficial microorganisms may represent a reliable alternative to reduce the application of pesticides in modern agriculture. Combination of microorganisms having multiple positives and complementary functions could be more beneficial for increasing growth and yield and may provide plant protection under different conditions (Harman, 2000). Thus, a better understanding of the interactions between beneficial microorganisms may help in the development of sustainable agricultural practices.

A number of studies have reported the interaction of AM fungi with *Trichoderma* sp. The major results of this interaction is reviewed and presented in Table 2. Co-inoculation of AM fungi with *Trichoderma* sp. has been applied for several decades for their synergetic anticipated effects on plant growth and disease suppression (Kohl and Schlosser, 1989; Haggag and Abd-El Latif, 2001; Srivastava *et al.*, 2010).

Table 2. Interaction effects of arbuscular mycorrhizal (AM) fungi with *Trichoderma* species (modified from Saldajeno *et al.,* 2008)

AM fungus	*Trichoderma* species	Experimental condition	Inoculation timing	Interaction effects	References
Glomus etunicatum	*T. hamateum* *T. harzianum*	soil	T=AMF	No effect on AM fungi root colonization on maize	Kohl and Schlosser, 1989
G. etunicatum *G. mosseae*	*Gliocladium virens**	soil	T=AMF	Effects of T on AM fungi colonization were variable on cucumber depending on medium used	Paulitz and Linderman, 1991
G. mosseae	*T. harzianum* *T. aureoviride*	*in vitro*	AMF=T	No effect on germination of AM fungi resting spores; enhanced production of AM fungi vegetative spores	Calvet *et al.*, 1992
G. mosseae	*T. harzianum*	soil	AMF>T	Decrease of AM fungi root colonization on soybean	Wyss *et al.*, 1992
G. mosseae	*T. aureoviride* *T. harzianum*	Peat-perlite mixture	AMF>T	Increased AM funfi root colonization on marigold; enhanced dry weight and foliar area	Calvet *et al.*, 1993
G. mosseae	*T. koningii*	*in vitro*	AMF=T	T inhibite the germination of AM fungi; no effect of AM mycelial development	McAllister *et al.*, 1994a
G. mosseae	*T. koningii*	soil	AMF<T AMF=T AMF>T	Variable effects on AM fungi colonization; T population in soil and plant dependent on time of inoculation on lettuce and maize	McAllister *et al.*, 1994b

(continued)

Gi. Gigantea	*Trichoderma* sp.	sand		Parasitism of AM fungi spores	Lee and Koske, 1994
G. intraradices	*T. aureoviride*	organic substrates	AMF>T	Increased plant growth of Citrus no effect of AM fungi root colonization	Camprubi *et al.*, 1995
G. intraradices	*T. harzianum*	soil	AMF=T	Significant decrease in severity and incidence of disease on tomato	Datnoff *et al.*, 1995
G. intraradices	*T. harzianum*	*in vitro*	AMF<T	Parasitism of extraradical mycelium of AM fungi	Rouseau *et al.*, 1996
G. mosseae	*T. harzianum*	soil	AMF=T	Negative or no effect on root colonization of AM fungi; increased SDW and height of pigeon pea; reduced nematode population and wilting index	Siddiqui and Mahmood, 1996
G. mosseae	*T. pseudokoningii*	*in vitro* soil	AMF=T AMF<T	No effect on AM fungi spore germination and hyphal growth; no effect of AM Fungi root colonization of *Gliocladium roseum;* increased population of *T. pseudokoningii*; decreased population of *T. harzianum*	Fracchia *et al.*, 1998

(continued)

G. intraradices	*T. harzianum*	*in vitro*	AMF<T	Conidial germination of T were stimulated in the presence of the AM fungal extract	Filion *et al.*, 1999
G. intraradices	*T. harzianum*	soil	AMF<T	No effect on AM fungi P transport; adverse effect on SDW; effect on RDW; reduced root colonization by AM fungi	Green *et al.*, 1999
G. deserticola	*T. harzianum*	soil	AMF=T	No effect on AM fungi or T colonization; no effect on the growth of maize	Vázquez *et al.*, 2000
G. mosseae	*T. harzianum*	*in vitro* soil	AMF=T	Enhanced AM fungi growth in dual cultures; increased T population in rhizosphere soil; increased growth of Geranium; reduced root-rot	Haggag and Abdel Latif, 2001
Gi. rosea *G. mosseae*	*T. pseudokoningii* (several strains)	*in vitro*	AMF=T	T inhibe spore germination of both AM fungi	Martinez *et al.*, 2004
G. fasciculatum	*T. harzianum*	soil	AMF=T	Effect on plant growth and transpiration of chickpea plant with and without plant pathogens; no effect of AM fungi root colonization.	Siddiqui and Singh, 2004

(continued)

G. mosseae	*T. harzianum*	peat	AMF=T	No effect on the CFU of T; no effect of AM root colonization of strawberry; no effect on plant growth; decreased the number of oospores of *Phytophotora fragaria*	Vestberg *et al.*, 2004
G. deserticola *G. mosseae*	*T. koningii*	soil	AMF=T	Increased SDW, nutrient concentration and chlorophyll; content of *Eucalyptus globulus* in contaminated soil; increased percentage of AM root length colonization	Arriagada et *al.*, 2005, 2007
G. mosseae	*T. pseudokoningii*	soil	AMF=T	Suppressed nematode reproduction and reduced nematode galling;	Oyekanmi *et al.*, 2007
G. intraradices	*T. harzianum* *G. virens**	soil	AMF=T	Increased in the growth of tomato plants; Reduction in galling and nematode multiplication	Siddiqui and Akhtar, 2008
G. mosseae	*T. harzianum*	soil/sand	AMF=T	Enhanced cucumber plant growth; increased the percentage of AM fungi root colonization; decreased the population development of T; suppressed damping-off disease	Chandanie *et al.* 2009

(continued)

G. intraradices *G. mosseae* *G. claroideum* *G. constrictum*	*T. harzianum*	soil	AMF=T	Decreased melon fresh weight; increased the percentage of AM fungi root colonization; decreased the population development of T; suppressed plant pathogen *Fusarium*	Martinez-Media *et al.*, 2009
G. intraradices	*T. harzianum*	soil	AMF=T	Reduced the incidence of *Fusarium* wilt of tomato; Increased tomato yield	Srivastava *et al.*, 2010

SDW = shoot dry weight; RDW = root dry weight; AM fungi = arbuscular mycorrhizal fungi; T = *Trichoderma*; CFU = colony forming unit. AMF<T = arbuscular mycorrhizal fungi inoculated before *Trichoderma* sp.; AMF=T = arbuscular mycorrhizal fungi inoculated at the same time than *Trichoderma* sp.; AMF>T = arbuscular mycorrhizal fungi inoculated after *Trichoderma* sp.

* The morphological borders between *Trichoderma* and *Gliocladium* are blurred. However, according to results obtained by molecular methods, *Gliocladium virens* is now generally recognized as belonging to the genus *Trichoderma* (Gams and Bisset 1998)

Even though their synergistic or additive effect have been reported on growth and yield and for the control of various plant pathogens (Calvet *et al.*, 1993; Datnoff *et al.*, 1995; Chandanie *et al.*, 2009), a number of studies also reported negative effects on the AM development (Rousseau *et al.*, 1996; Green *et al.*, 1999; Martinez *et al.*, 2004) and a concomitant reduction in plant performance (*i.e.* shoot and root dry weights) (McAllister *et al.*, 1994a, b; Martinez *et al.*, 2004).

The antagonistic mechanisms of *Trichoderma* sp. reported on plant fungal pathogen may pose risks to the non-target AM fungi. For instance, McAllister *et al.* (1994a) and Martinez *et al.* (2004) observed that the saprotrophic fungi produce soluble and volatile substances affecting AM spore germination. Metabolites produced (*i.e.* chitinase) by *T. harzianum* may also play a role in mycoparasitism of AM fungi (Rousseau *et al.*, 1996). *Trichoderma* sp. has been shown to penetrate spores (Lee and Koske, 1994) and extraradical mycelium of the AM fungi, resulting in dissolution of the cell walls (Rousseau *et al.*, 1996). These saprotrophic fungi can also affect the development of the mycorrhizal presymbiotic phase (Wyss *et al.*, 1992; McAllister *et al.*, 1994b) and decrease AM fungi root colonisation, possibly through interaction with the external mycelium of the mycorrhizal fungus (Green *et al.*, 1999). Wyss *et al.*, 1992 attributed the reduction of mycorrhizal root colonization to the induction of plant defence compound (*i.e.* an antimicrobial phytoalexin) in root tissues in presence of *T. harzianum*. The ability of *Trichoderma* sp. to impede

colonization of plant roots by AM fungal species appears to be dependent on the order of inoculation of the fungi. McAllister *et al.* (1994b) demonstrated that root colonization by *G. mosseae* was reduced only when plants were simultaneously inoculated with *T. koningii* and *G. mosseae* or when *T. koningii* was inoculated before the AM fungus. The presence of *T. koningii* affected the AM fungi metabolic activity, materialized in a decrease in the amount of *G. mosseae* hyphae showing succinate dehydrogenase activity (McAllister *et al.*, 1994b). Also, in the presence of AM fungi, the population of *T. harzianum* in the rhizosphere was reduced indicating that the AM fungus appeared to be antagonistic to saprotrophic fungi (Fracchia *et al.*, 1998; Green *et al.*, 1999).

According to the diversity of results obtained, there are clear evidences that the interaction between AM fungi and *Trichoderma* sp. may produce a different effect (*i.e.* synergistic to antagonistic) on plant growth and fungal development depending amongst other things on the genus considered as well as on the environmental condition. Since interactions of biocontrol agents with beneficial organisms in the rhizosphere might stimulate biocontrol in an agroecosystem (Calvet *et al.*, 1993; Paulitz and Linderman, 1991; Brimmer and Boland, 2003), it is important to study interactions of biocontrol agents with non-target beneficial organisms in the soil to ensure the successful development and usage of biocontrol strategies.

III. RESEARCH OBJECTIVES

Research objectives

The combination of AM fungi with *T. harzianum* to improve plant health and increase plant productivity may represent an alternative to the application of pesticides and fertilizers in sustainable agriculture (Saldajeno *et al.*, 2008). However, results published so far are controversial. *T. harzianum* is known to be an opportunistic, avirulent plant colonizer but has also been reported as antagonist of other fungi including the AM fungi. Thus, it appears essential to understand the mechanisms which govern the interaction between *T. harzianum* and *Glomus* sp. in order to manage these microorganisms in the most efficient way to improve plant health and productivity.

The main objective of this thesis was to study the interaction of *T. harzianum* (MUCL 29707) with an AM fungus, *Glomus* sp. (MUCL 41833) in order to develop a microbial inoculum containing both beneficial microorganisms.

Therefore, different question were asked:

1. Is it possible to extend the classical *in vitro* culture systems used with single microbes (*i.e.* AM fungi), to multiple microbes (*i.e. Glomus* sp., *Rhizoctonia solani* and *T. harzianum*)?

2. How do *T. harzianum* affect the AM symbiosis?

2.1. Do *T. harzianum* impact the development and the viability of *Glomus* sp.?

2.2. Do *T. harzianum* decrease the P transport by *Glomus* sp.?

3. Does co-entrapment of *Glomus* sp. with *T. harzianum* fungi within the same alginate inoculum affect the germination and establishment of the AM symbiosis?

IV. CHAPTER 1

Coupling autotrophic *in vitro* plant cultivation system to scanning electron microscope to study plant-fungal interactions

Spanish Journal of Agricultural Research (2010) 8(S1): S69-S78

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1. Preface

Investigating and understanding the interactions between symbiotic (AM fungi) and saprotrophic (*e.g. T. harzianum*) microorganisms necessitates the development of adequate culture conditions to separate the direct interactions from potentially misleading abiotic or biotic factor. Here, the HAM-P culture system developed for the *in vitro* mycorrhization of micropropagated potato plants (Voets *et al.*, 2005) was extended and adapted to study the life cycle of three major soil microorganisms: a symbiont (*Glomus* sp.), a saprotroph (*T. harzianum*) and a plant pathogen (*Rhizoctonia solani*). The life cycle of the three organisms was described on their common host, the potato, under strict controlled conditions.

2. Summary

The interactions of plants with pathogens and beneficial micro-organisms have been seldom compared on the same host and under strict controlled autotrophic *in vitro* culture conditions. Here, the life cycle of two plant beneficial (*Glomus* sp. MUCL 41833 and *Trichoderma harzianum*) and one plant pathogen (*Rhizoctonia solani*) fungi were described on potato (*Solanum tuberosum*) plantlets under autotrophic *in vitro* culture conditions using video camera imaging and the scanning electron microscope (SEM). (i) The colony developmental pattern of the extraradical mycelium within the substrate, (ii) the reproduction structures and (iii) the three-dimensional spatial arrangements of the fungal hyphae within the potato root cells were successfully visualized, monitored and described. The combination of the autotrophic *in vitro* culture system and SEM represent a powerful tool for improving our knowledge on the dynamics of plant-fungal interactions.

Keywords: arbuscular mycorrhizal fungi, autotrophic culture system, potato, *Rhizoctonia*, scanning electron microscope, *Trichoderma*.

3. Introduction

The rhizosphere (*i.e.* zone of soil influenced by plant roots and characterized by an important microbiological activity) represents a highly dynamic region governed by a complex mosaic of interactions between plants and microorganisms (Kennedy and De Luna, 2004). Some microorganisms inhabiting this zone are beneficial to plants (*e.g.* increase soil fertility and structure, control root pathogens) (Barea *et al.*, 2005), while others are detrimental (*i.e.* pathogenic) causing considerable damage to economical important crops (Higa and Parr, 1994). One primary objective in studies of the biology of this soil-root interface is to improve the interactions between the plant and the plant-beneficial microorganisms (*e.g.* arbuscular mycorrhizal (AM) fungi, *Trichoderma* species), whilst controlling the harmful effects of those which are phytopathogenic.

Host-pathogen interactions as well as host-beneficial microorganisms interactions are multi-step processes that involve adhesion to the host surface, infection/colonization of root tissues, which provokes cell damage and disease or help the plants to acquire nutrients and withstand stress conditions. To study these processes several *in vivo* and *in vitro* systems have been set up that includes both the host and the pathogenic or beneficial micro-organisms (Friese and Allen, 1991; Olivain and Alabouvette, 1999; Vleeshouwers *et al.*, 1999; Declerck *et al.*, 2005; Huang *et al.*, 2005). Recently, an autotrophic culture system was developed for the *in vitro*

mycorrhization of micropropagated potato (*Solanum tuberosum*) plantlets (Voets *et al.*, 2005). This system provides unique visualization of the AM fungal development and the possibility to conduct time-course non-perturbed three-dimensional morphological observations.

The potato has been described to form beneficial associations with AM fungi (Duffy and Cassells, 2000) and *Trichoderma* spp. (Harman *et al.*, 2004), while being severely affected by *Rhizoctonia solani* (Sneh *et al.*, 1996). Arbuscular mycorrhizal (AM) fungi and fungi from the genus *Trichoderma* are well known to develop beneficial associations with plants (Harman *et al.*, 2004; Smith and Read, 2008). Members of both groups increase plant growth and productivity and have been shown to behave as biocontrol agents against a wide range of important soil-borne plant pathogens (Harman *et al.*, 2004; Barea *et al.*, 2005). Certain members of the *Rhizoctonia* genus (*i.e. R. solani*) (Sneh *et al.*, 1996) are known to be pathogens causing serious damages to some of the most important economical crops throughout the world.

Although the life cycles of many soil-borne fungi have been studied *in vitro*, most have not been compared *in vivo* on a single host plant species (*e.g.* potato) that is susceptible to a large number of pathogenic (*e.g. R. solani*) and beneficial (*e.g.* AM fungi and *Trichoderma*) microorganisms. This difficulty was partly related to the obligate symbiotic nature of AM fungi which, *in vitro*, was most

often studied on transgenic excised roots, *i.e*. without photosynthetic tissues (see Declerck *et al.*, 2005). Hence, the objective of this work was to describe *in vitro* the life cycle of three major fungi (*i.e. Glomus* sp. MUCL 41833, *T. harzianum* and *R. solani*) on autotrophic potato plantlets as a single host. For the three fungi, the hyphal extension, colonization of the substrate, root infection/colonization and reproduction structures were described under autotrophic *in vitro* culture conditions (Voets *et al.*, 2005), combining the video camera imaging (de la Providencia *et al.*, 2005) and the cryo-electron scanning microscope (cryo-SEM) (Read and Jeffree, 1991).

4. Material and methods

4.1. Biological material

4.1.1. Propagation and maintenance of potato plantlets stock

In vitro propagated potato plantlets (*Solanum tuberosum* L. cv. Bintje) were supplied by the Station de Haute Belgique in Libramont, Belgium. Plantlets were micro-propagated every five weeks by placing nodal cuttings in sterile culture boxes (90 × 60 × 50 mm, 20 cuttings per box) (see Voets *et al.*, 2005), filled with 50 ml of 4.41 g L–1 sterilized (121°C for 15 min) Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), supplemented with 20 g l^{-1} sucrose, 3 g l^{-1} Gel Gro™ (ICN, Biomedicals, Inc., Irvine, CA, USA) and adjusted to pH 5.9. Plantlets were kept in a growth chamber at 20/15°C (day/night) and illuminated for 16 h d^{-1} under a photosynthetic photon flux of 15 μmol m^{-2} s^{-1}.

4.1.2. Culture, propagation and maintenance of *Glomus* sp. MUCL 41833, *Trichoderma harzianum*, and *Rhizoctonia solani*

The AM fungus used was *Glomus* sp. MUCL 41833, formerly identified as *Glomus intraradices* and presently reclassified in a clade that contains the recently described species *G. irregular* Blaszk., Wubert, Renker and Busco (Stockinger *et al.*, 2009) was supplied by Glomeromycota *IN vitro* Collection (GINCO) (http:// www.mbla.ucl.ac.be/ginco-bel). The spores were extracted by

solubilization of the gellan gel (Doner and Bécard, 1991) and an approximate of 100 were placed close to actively growing transformed carrot (*Daucus carota* L.) roots (approximately 70 mm length) on Petri plates (90 mm diam.) containing the Modified Strullu-Romand (MSR) medium (Declerck *et al.*, 1998 modified from Strullu and Romand, 1986) solidified with 3 g l^{-1} Gel Gro (ICN, Biomedicals, Inc., Irvine, CA, USA) (Cranenbrouck *et al.*, 2005). The Petri plates were incubated during 3 months in the dark in an inverted position at 27°C. Several thousand spores and extensive mycelium were produced within this period.

A culture of *Trichoderma harzianum* Rifai MUCL 29707 was supplied by the Mycothèque de l'Université catholique de Louvain (MUCL) (http://bccm.belspo.be/about/mucl.php). A plug of gel containing several conidia and mycelium was placed into a sterile 1.5 ml glass tube filled with 0.4 ml of 1% sterile distilled waterpeptone (SDWP) (Duchefa, The Netherlands). The plug was swirled in the SDWP with a vortex mixer for 15 s and the suspension was serially diluted to 10^{-2}. From the 10^{-2} dilution, 50 µl was spread on potato dextrose agar (PDA) (Scharlau Chemie S.A, Barcelona, Spain) Petri plates and further incubated at 25°C for seven days. The Petri plates were subsequently maintained at 4°C until needed.

A culture of *Rhizoctonia solani*[1] Kuhn MUCL 49235 was supplied by MUCL. A plug of gel containing several bulbils (or

[1] Anastomosis group 3 (AG 3)

sclerotia, hard pieces of mycelium tissue) (Sneh *et al.*, 1996) and mycelium was placed on PDA Petri plates that were then incubated at 25°C for seven days.

4.2. Experimental set-up

Ten days old potato plantlets were used to study the life cycle of the three fungal organisms under *in vitro* autotrophic culture conditions. One potato plantlet was inserted in each Petri plate on the MSR medium, with the roots placed on the surface of the medium and the shoot extending outside through the hole made at the base and the lid of the Petri plate (for details, see Voets *et al.*, 2005). Spores of the AM fungus cultures were isolated by solubilization of the MSR medium (Donner and Bécard, 1991) and maintained in deionized sterilized (121°C for 15 min) water before inoculation. Approximately 50 spores were placed in the vicinity of the roots of potato plantlets in each Petri plate. The Petri plates were then closed with parafilm^{TM} (Pechiney, Plastic Packaging, Chicago) and the hole cautiously plastered with sterilized (121°C for 15 min) silicon grease (VWR International, Belgium) to avoid contaminations (Voets *et al.*, 2005).

The inoculation with the *T. harzianum* strain was performed using 50 µl of fungal suspension from a 10^{-2} dilution containing several conidia and mycelium (see above). The suspension was spread on Petri plates containing PDA and incubated at 25°C for 24 h. A

single-germinated conidium was transferred to the root vicinity of potato plantlet and the Petri plates were closed as above.

The inoculation with *R. solani* was performed with a 3 mm diameter plug removed with a sterile cork borer from a seven day old culture on PDA medium. The plug containing abundant mycelium and bulbils was placed in the vicinity of roots of potato plantlets and the Petri plates were closed as above.

Whatever the fungus, all the Petri plates were covered with an opaque plastic bag and incubated horizontally in a growth chamber set at 22/18°C (day/night) with 70% relative humidity, a 16 h photoperiod and a photosynthetic photon flux (PPF) of 300 µmol m^{-2} s^{-1}. Ten ml of sterilized (121°C for 15 min) MSR medium lacking sucrose and vitamins (cooled to 40°C in a water bath) was added every two weeks in the Petri plates containing the AM fungus to maintain an adequate level of MSR medium and to supply the plantlets with renewed nutrients. For each fungus, five replicates were examined. In addition, five uninoculated potato plants were used as control.

4.3. Observations and description of the fungal development

For the three fungi, the hyphal extension, colonization of the substrate, root infection/colonization and reproduction structures were described. Anastomosis, that is *«a mechanism by which different branches of the same or different hyphae fuse to constitute a mycelial*

network» (Kirk *et al.*, 2001) formed during the colony development of the three organisms was further categorized following the detailed description performed by Buller (1933). For the AM fungus the Petri plates were checked weekly during 6 weeks following the method described in Declerck *et al.* (2001). For *T. harzianum* and *R. solani* strains the development of the fungal colony was followed daily during 2 weeks due to the high speed of mycelium development. All the measurements were conducted under a dissecting microscope (Olympus SZ40, Olympus Optical GmbH, Hamburg, Germany) (magnification x6.7 to x40) and compound bright-field light microscope (Olympus BH2-RFCA, Olympus Optical GmbH, Hamburg, Germany) (magnification x50 to x250). Images of the different stages of the life cycle of the three fungi were captured with a digital camera (model Leica DFC320; Leica Microsystems Ltd.) coupled to a compound brightfield light microscope Olympus BH2-RFCA and displayed on a 15-inch hp pavilion ze4500 computer using the image manager software: Leica IM50, version 4.0 Leica Microsystems Imaging solutions Ltd., Cambridge, UK.

Newly produced spores of *Glomus* sp. MUCL 41833, conidia of *T. harzianum* and mycelium and bulbils of *R. solani*, isolated from autotrophic *in vitro* cultivation system after 6 weeks and 12 days, respectively, were tested for their capacity to germinate and ability to produce the fungal life cycle. Spores of the AM fungus were isolated by solubilization of the MSR medium (see above) and placed in the vicinity of roots of potato plantlets in Petri plates (90 mm diam.)

containing the MSR medium without sucrose and vitamins (see above). The Petri plates were incubated in the same condition that described above. Germination was observed under stereo microscope at 10-40x magnification after 10 days. A plug of gel containing several conidia and mycelium of *T. harzianum* and a plug of gel containing several bulbils and mycelium of *R. solani* were placed on PDA and MSR Petri plates and further incubated at 25°C for three days. The regrowth of *T. harzianum* and *R. solani* was observed under stereo microscope at 10-40x magnification.

4.4. Tissue preparation and cryo-fracturing electron scanning microscopy (cryo-SEM)

Six weeks after inoculation with the AM fungus and 6 and 12 days after inoculation with *T. harzianum* and *R. solani*, some of the roots developing on the surface of the MSR medium and in contact with the fungal mycelium were gently removed from the gel with forceps without damaging the whole root system and disturbing the fungal colony development. Six days old, 12 days old and 6 week old un-inoculated roots of potato plantlets were also gently removed from the gel and used as control for comparing the level of damage caused by the pathogens with the normal process of root aging. The roots were excised to 1 cm length and put into sterilized distilled water. The root pieces were subsequently transferred to Press-to-Seal™ (Molecular Probes) silicone chambers and pressed onto a dry microscope slide 24 × 60 mm (Menzel-Glazer®). Sterilized wateragar 3% (0.6 ml) (Oxoid,

Basingstoke, Hampshire, England) was added to the chamber to fix and immobilize the root pieces, and thus to create a thin layer in which they were embedded. Subsequently, the silicone isolator chamber was removed and the root pieces embedded in the solid water-agar were transversally cut with a sterile scalpel. For SEM observations (SEM Phillips XL20, Amsterdam, The Netherlands), roots were flash frozen (–212°C) in liquid nitrogen under vacuum for cryo-SEM (Oxford CT1500 cryosystem, Oxford, UK), transferred to the preparation chamber and then to the SEM chamber where the frozen samples were sublimated (–80°C) to remove ice particles. Samples were viewed under 2-5 kV at –170°C to –190°C.

5. Results

5.1. *Glomus* sp. MUCL 41833 development

Germination of the AM fungus spores was observed two days after inoculation. Straight, coenocytic and hyaline germinating hyphae arose through the subtending hyphae of spores and developed on the surface of the medium. Vigorous 10-15 μm wide hyphae branched at angles of approximately 45° and extended over short distances on the MSR medium. Following contact with the host root (within one week) several appressoria[1] were formed (Fig. 7a). Subsequently, multiple hyphae developed intercellularly within the cortex (Fig. 7b) extending root colonization beyond the site of penetration. In parallel, runner hyphae (RH) developed extensively in the growth medium, and ramified repeatedly into hyphae bearing numerous branching absorbing structures (BAS). Interconnections of hyphae by means of hyphal fusions (anastomosis) were often observed in all parts of the colony. Fusions between hyphae were observed through tip-to-tip or tip-to-peg fusions to form an intermingled network of mycelium.

[1] Renamed hyphopodia (Bonfante and Genre, 2010)

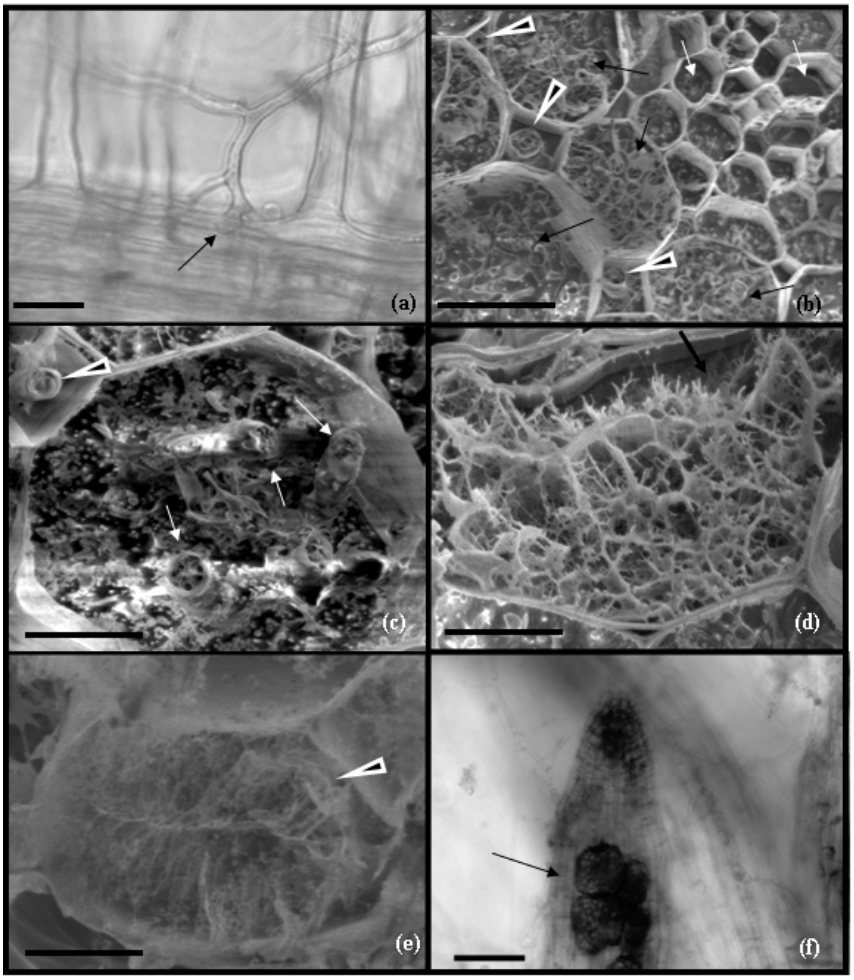

Figure 7 Description of the development of *Glomus* sp. MUCL 41833 under autotrophic conditions in association with *Solanum tuberosum.* (a) Appressorium[1] formation on the surface of the host root (black arrow), bar = 100 µm. (b) Intercellular development and spreading of hyphae (white arrowhead). Note that the sequestered solutes during freezing give the striated appearance to the fully turgescent root cells (black arrow). Some cells are empty caused by the aging process of cell tissue (white arrow), bar = 50 µm. (c) Sectioned root cell showing the development of the 1st order branches of arbuscule within the cortical cell (white arrows) as well as the intercellular hyphal spreading of the fungi (white arrowhead), bar = 30 µm. (d) Full developed and highly branched *Arum*-type arbuscule (black

[1] Renamed hyphopodium (Bonfante and Genre, 2010)

arrow). Note that the arbuscule is occupying the entire volume of the root cell, bar = 40 μm. (e) Senescing arbuscule (white arrowhead), bar = 40 μm. (f) Formation of vesicle and spores a few millimetres behind the apex root zone (arrow), bar = 60 μm.

The first daughter spores appeared four weeks post-inoculation and their formation was observed mainly on the primary hyphae (*i.e.* RH). After extensive colonization of the substrate (*i.e.* eight weeks post-inoculation) hundreds of spores of 92.7 ± 2 μm diam. were produced. Numerous vesicles and intraradical spores were observed within roots. Some of them were observed a few millimetres behind the root apex (Fig. 7f). Observation of the root under cryo-SEM six weeks post-inoculation revealed the structural details of the development of the *Arum*-type arbuscule, intercellular hyphae penetrate cell walls at the inner root cortex and ramify in the apoplast finally forming one highly branched tree-like structure (Smith and Read, 2008) (Fig. 7c-e). Sectioned root segments showed the 1st order branches of arbuscules (Fig. 7c), highly branched mature arbuscules, occupying the entire cell volume (Fig. 7d) and degraded arbuscules (Fig. 7e). Root cells in intimate contact with the AM fungus did not show any signs of cellular degradation, but fully turgescent cells were often observed showing striations which are sequestered solutes formed during freezing (Fig. 7b). Cryo-SEM analysis after six weeks of culture also showed empty root cells caused by the aging process of the root cell tissues (Fig. 7b).

The observations of spore germination showed that 93% of the newly produced spores germinated within 10 days. Hyphae growth

was observed emerging through the lumen of the subtending hyphae. The spores were able to form associations with the roots of the plantlets in the autotrophic *in vitro* culture system and produce new spores.

5.2. *Trichoderma harzianum* MUCL 29707 development

The development of *T. harzianum* was marked by a rapid germination of conidia. Highly branched hyaline germinating hyphae, branched at angles from 10 to 90°, extended rapidly on the MSR medium. During the first 24 h, hyphae (3-10 μm diam.) extended radially and a dense network of interconnected hyphae was observed in all parts of the colony where anastomoses were observed through to tip-to-tip, tip-to-peg and peg-to-peg fusions. Forty-eight hours later (72 hours post-inoculation) the entire Petri plates (*i.e.* roots and MSR medium) were covered with the fungal mycelium (Fig. 8a). Contacts with roots were observed (Fig. 8b) at that time. Aerial mycelium with arachnoid's appearance was coiled to the aerial roots (Fig. 8c). However, the direct and scrupulous observations of roots under bright-field light and electron scanning microscopes did not reveal any structure resembling appressorium. Five days after inoculation, conidiogenous cells (phialides) producing single phialidospores at the tip of each phialide were observed (Fig. 8d). Conidiophores were gathered in loose aggregates scattered on the surface of the medium or forming compact tufts along and attached to the roots (Fig. 8e).

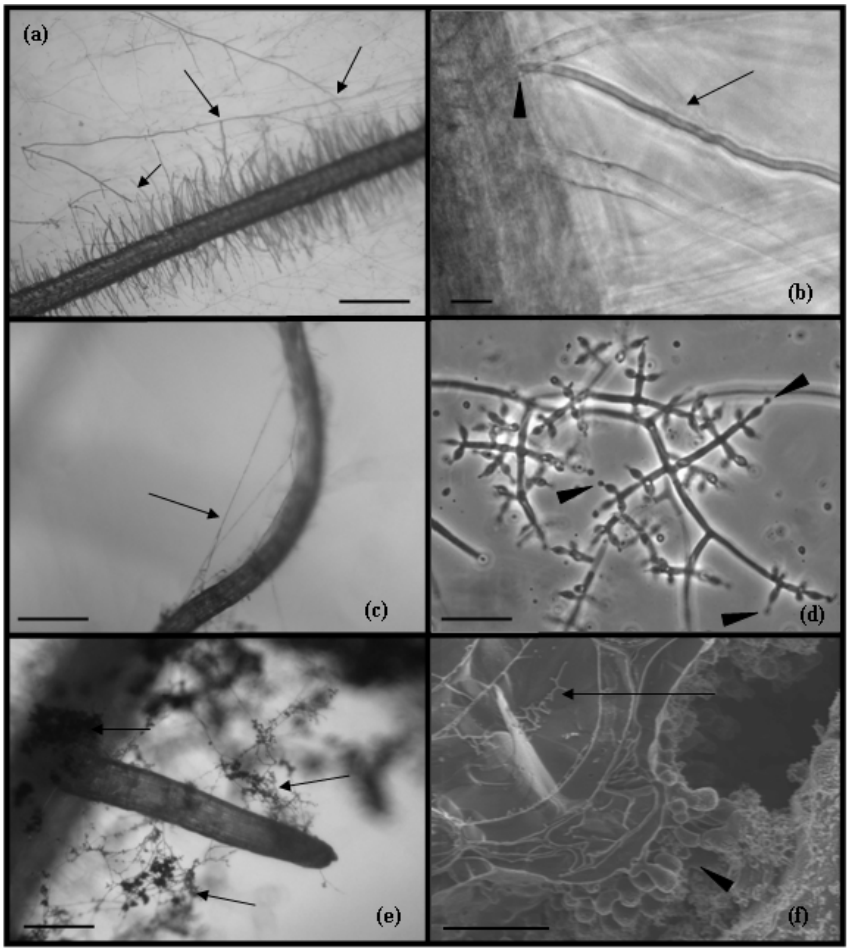

Figure 8 Description of the development of *Trichoderma harzianum* MUCL 29707 under autotrophic conditions in association with *Solanum tuberosum*. (a) Highly ramified mycelium (black arrows) spreading and colonizing the substrate (MSR medium), bar = 250 µm. (b) Growth of the hypha towards the root (arrow). Note the contact with the surface of the root (arrowhead), bar = 30 µm. (c) Aerial mycelium coiled around the roots (arrow), bar = 250 µm. (d) Conidiogenous cells producing single phialidospores at the tip of each phialide (black arrowheads), bar = 25 µm. (e) Conidiophores forming compact tufts along and attached to the roots (arrows), bar = 200 µm. (f) Sectioned root segment showing an intact cortical cell (arrow). Note the presence of a mature conidiophore attached to the root surface (arrowhead), bar = 20 µm.

The distribution of the conidiophores along the root axis was not correlated with the age of the root, since these structures were observed along the main root as well as in secondary and lower order branches. Gradual changes in the colour of the aggregates or tufts from white to green and finally greyish-green, following production and maturation of the conidia, were observed. No chlamydospores were detected during the experimental period.

No root cells invasion was observed under SEM after 6 days or after 12 days of roots contact. Hyphal development as well as conidiophores formation was confined to the epidermis root zone (Fig. 8f). The root cells did not show any signs of degradation caused by the presence of *T. harzianum*. Full content striated cells caused by cryo-preservation process were observed. The mycelium and conidia of *T. harzianum* produced in the autotrophic culture system were able to regrowth and to produce new conidia.

5.3. *Rhizoctonia solani* MUCL 49235 development

Within the initial 24 hours following inoculation several hyaline hyphae emerged from the plug. Hyphae close to the inoculation plug presented a negative geotropism (hyphae growing upward, contrary to the gravity) and were highly ramified. The mycelium consisted of septate, hyaline hyphae, branching at 45-90° angles.

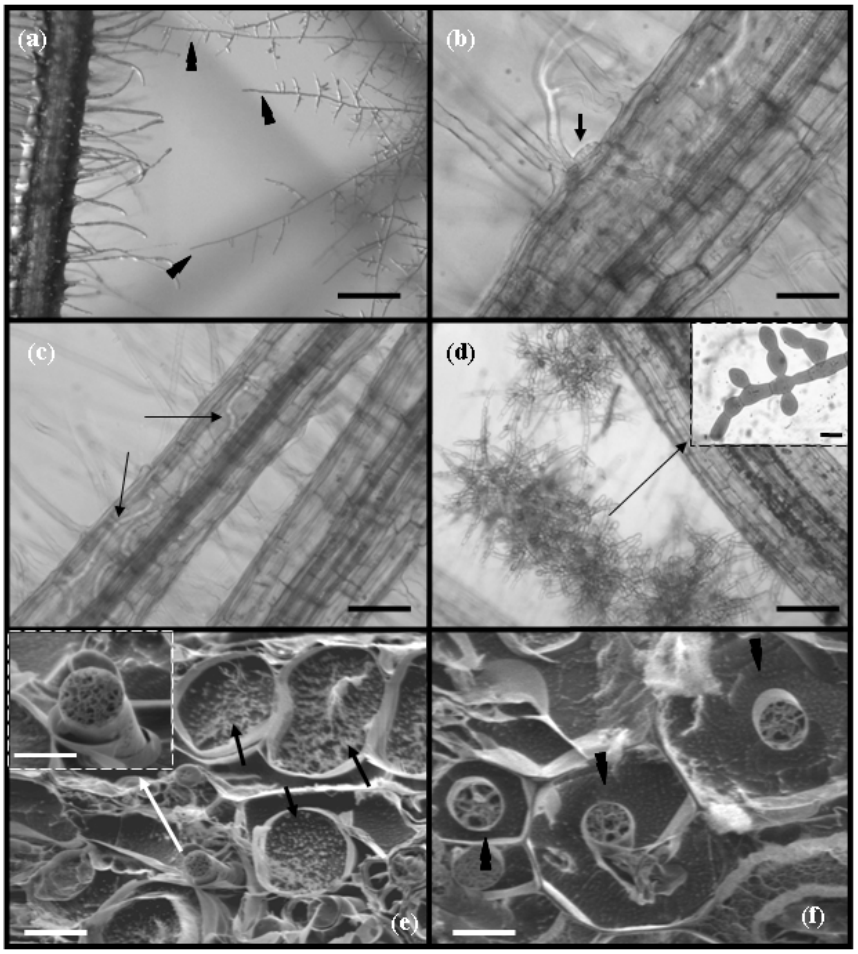

Figure 9 Description of the development of *Rhizoctonia solani* MUCL 49235 under autotrophic conditions in association with *Solanum tuberosum*. (a) Highly branched hyphae growing towards the roots (double arrowheads), bar = 250 µm. (b) Contact with root and formation of a T-shaped branch" appressorial structure at the surface of the root (arrow), bar = 50 µm. (c) Intracelullar spreading of hyphae. Note that the spreading of hyphae is parallel to the root vertical axis (arrows), bar = 200 µm. (d) Formation of compact tufts of bulbils (reproduction structures) close to the roots, bar= 200 µm. Inset: Closer view of bulbils, bar = 10 µm. (e) Highly deteriorated cortical cells (black arrow) due to the presence of *R. solani* hyphae, bar = 15 µm. Inset: Closer view of intercellular hypha, bar = 5 µm. (f) Well-preserved root cells being infected by *R. solani* hyphae (double arrowheads). Note that the root cells did not collapse due to the presence of intracellular hyphae, bar = 8 µm.

The hyphal width varied from 5 to 12 μm. The mycelium was growing over the surface of the medium and no contact with roots was observed within this period. Forty-eight hours later (72 hours post-inoculation) the mycelium developed extensively and hyphae were growing into the medium. Hyphal anastomoses involving tip-to-tip and tip-to-peg fusions were clearly observed in all parts of the colony. Long hyaline hyphae having numerous lateral branches were growing towards the adjacent roots and colonizing the substrate (Fig. 9a). Hyphal swelling and coiling forming a right angle branch called «T-shaped branch» and giving rise to short appressorial structure or infection pegs, were observed on several points of the root surface (Fig. 9b).

No formation of infection cushions was observed after inoculation during the whole experiment. The mycelium was firmly attached to the root surface. Subsequently, hyphae penetrated the epidermal tissue and developed both inter- and intracellularly, parallel to the root vertical axis (Fig. 9c). Necrosis of the root hairs near the infection points was the first sign of fungal damage. However the observations under bright-field light and scanning electron microscopes did not reveal fungal infection via the root hairs nor fungal occupation in this root zone. Particularly, the cryo-SEM observations of the roots 6 days after inoculation showed hyphal invasion of cortical cells. Total degradation of cell cytoplasm caused by the pathogen was observed (Fig. 9e). However deterioration of the root tissue was not extended to the whole root system.

Undifferentiated aggregations of thick-walled melanized cells called bulbils, that represent the survival structures of *R. solani*, were observed after seven days of inoculation (Fig. 9d). These structures were observed mainly superficially (Fig. 9d) or detected attached to the roots. The cryo-SEM observations twelve days after inoculation revealed severe damage to the entire root system. Even when the cytoplasmic content of the root cells was totally degraded at that time (Fig. 9e), our observations showed well-preserved cell walls (*i.e.* without deformation or distortion in their structure). Fungal hyphae were easily observed within the root cells (Fig. 9e) and in the intercellular spaces. In addition, hyphae were mostly aligned with the vertical root axis (Fig. 9f). The mycelium and conidia of *R. solani* produced in the autotrophic culture system were able to regrow and to produce new bulbils.

6. Discussion

The development of appropriate *in vitro* autotrophic plant cultivation systems on which several fungi with different relationships to root cells (*i.e.* obligate symbiont, non-obligate plant-beneficial fungi, and pathogen) can develop represents an important tool to study plant micro-organisms interactions. Here, we successfully monitored, visualized and described the life cycle of *Glomus* sp. MUCL 41833, *T. harzianum* MUCL 29707 and *R. solani* MUCL 49235 on potato plantlets grown *in vitro* under autotrophic culture conditions. The system allowed for easy visualization of the fungal structures by means of cryo-SEM analysis performed on root cells in direct contact with these fungi.

The life cycle of *Glomeromycota* (Schüßler *et al.*, 2001) has been described for several species (Fortin *et al.*, 2002). The development of root organ culture (ROC) (see Declerck *et al.*, 2005) improved significantly the description of the life cycle, among others for the vesicles-forming *Glomus* species (Chabot *et al.*, 1992; Strullu *et al.*, 1997). However, the use of ROC to study AM fungal symbiosis has some limitations, particularly the absence of photosynthetic tissues necessary to have a normal hormone balance and physiological source-sink relationships (Fortin *et al.*, 2002). To overcome this limitation, Voets *et al.* (2005) developed a system in which the plant roots were intimately associated to an AM fungus. In this system culture plant roots grew on a gelled medium under strict *in vitro*

conditions, while the photosynthetic shoot developed under open-air conditions. Using this system we observed spore germination, appressorium formation, root colonization, and spore production. In addition, SEM observations of the root cells revealed, for the first time, the inter and intraradical locations of hyphae and arbuscules development under *in vitro* conditions, supporting earlier observations made on *in vivo*-based experiments (see Smith and Read, 2008).

Arbuscules have been acclaimed as the major site for the exchange of minerals (in particular phosphorus) and carbohydrates between both partners in the AM symbiosis (Rosewarne *et al.*, 1999; Rausch *et al.*, 2001). Using a similar *in vitro* autotrophic cultivation system, Dupré de Boulois *et al.* (2006) demonstrated the transport of P from a hyphal compartment to a root compartment and subsequently to the plant shoot cells via the fungal interface, while the transport of carbohydrates from plant to the AM fungus was investigated by Voets *et al.* (2008). However, strict attention was not paid to either the development or presence of arbuscules. Therefore, the observations in our experiment of the presence, development and senescence of *Arum* type arbuscule under cryo-SEM coupled with the transport of P and C observed by Dupré de Boulois *et al.* (2006) and Voets *et al.* (2008), respectively, supported the role of arbuscule as an exchange interface and the adequacy of the autotrophic *in vitro* culture system to sustain this essential attribute of the symbiosis. The presence of vesicles and intraradical spores just behind the apex further demonstrated that AM fungi are able to penetrate the root at the apex zone (Bago and Cano,

2005) and also that intraradical structures such as vesicles may be located in this zone. These data supports the validity of the system used here to study AM fungal symbiosis under *in vitro* conditions.

The time-course monitoring of the life cycle of *T. harzianum* showed that the development of this strain was successfully completed under *in vitro* autotrophic culture conditions (*i.e.* from conidium germination to the formation of the reproduction structures). Numerous hyphae coiled around the roots and firmly attached to them with the formation of several mature conidiophores along the root system were observed. However, the scrupulous observations of roots under bright-field light microscope and cryo-SEM analysis on the root samples did not reveal any sign of fungal invasion in the root cortex.

Several studies have shown that root associated-*Trichoderma* strains resulted in increased levels of defense-related plant enzymes (see Harman *et al.*, 2004). These studies have related this phenomenon to the plant internal root colonization. However, currently only two reports have visualized the hyphal penetration of the roots by *Trichoderma* strains (Yedidia *et al.*, 1999; Chacón *et al.*, 2007). Yedidia *et al.* (1999) studied the induced defense mechanisms in cucumber plants (*Cucumis sativus* L.) following contact with *T. harzianum* T-203 (formerly re-described later as *T. asperellum* (Kullnig *et al.*, 2001). The electron microscopy analysis of ultrathin sections from *Trichoderma*-treated roots revealed penetration of *T. asperellum* hyphae into the roots mainly restricted to the epidermis

and outer cortex. However, in some localized areas, the hyphae were observed also in the endodermis (Yedidia *et al.*, 1999). Working with *T. harzianum* CECT 2413 in tobacco (*Nicotiana benthamiana*) and tomato plants (*Lycopersicum esculentum*) Chacón *et al.* (2007) demonstrated the ability of this fungus to colonize roots and to stimulate plant growth in seedlings of both species. Despite that our observations did not reveal fungal penetration and invasion with *T. harzianum* MUCL 29707 into the potato roots, our results are not in contradictions with the studies of Yedidia *et al.* (1999) and Chacón *et al.* (2007) since plants and fungal species/strain are different. Furthermore, *Trichoderma* species are not obligate biotrophs of plants but they are mostly saprotrophic on soil and dead plant material, but can also be opportunistic plant root colonizers (Harman *et al.*, 2004). Therefore, root colonization is not an essential attribute of this group of fungi. Nowadays, little is known about the mechanisms which govern this colonization. Further works should be addressed to study the effects of different host on the *«symbiotic behaviour»* of some *Trichoderma* strains (*e.g. T. asperellum* T-203, *T. harzianum* T-22 and T-39) that are largely known to produce defense responses in plants (Yedidia *et al.*, 1999, 2003; Harman *et al.*, 2004) and also the combination of both factors.

Studies of plant-pathogen interactions are often hampered by difficulties in obtaining undisturbed internal views of infected material (Refshauge *et al.*, 2006). The traditional methods used to study plant pathogen interactions drastically damage the arrangements of plant

and fungal cells (see Refshauge *et al.*, 2006), particularly when highly damaged tissue are sampled and prepared for microscopy observations and analysis (Chi and Childers, 1964; Ruppel, 1973). In our study, the life cycle of *R. solani* associated to potato plantlets was successful monitored and described. The system used here allowed for easy visualization of the spreading of the colony in the gelled medium and further monitoring of hyphae within roots. Hyphae were seen to propagate into the roots cells mainly following the direction of the root vertical axis. The same behaviour was earlier described in the root of cereals (Wenzel and McCully, 1991; Refshauge *et al.*, 2006). These authors suggested that the propensity of hyphae to colonize roots following the direction of the vertical axis is the result of a long, tubular structure of the cortical cells that provide the pathogen a lower resistance pathway for infection. The detection of hyphae of *R. solani* inside and between potato root cells as early as 6 days post-inoculation were similar to the findings of Refshauge *et al.* (2006) and Hall (1987). These authors detected hyphae of *R. solani* within roots of wheat (*Triticum aestivum*) after six and seven days respectively. Cryo-SEM analysis of highly damaged root cells caused by the necrophytic action of *R. solani* helps to overcome the difficulties to obtain good quality internal views of the delicate root system.

The autotrophic *in vitro* plant cultivation system and cryo-SEM analysis are adequate and important tools to study plant-fungi interactions, either beneficial or pathogenic. The ability to couple and to integrate both tools offers the possibility of deepening our

understanding on how soil-borne micro-organisms and plants behave during their interactions, giving a complete picture of the structural arrangements of the fungal cells within the root tissue during their whole life cycle. Hence, they are not limited to the study of plant-fungi but could be extended to other soil-borne micro-organisms that are largely known to develop beneficial and/or harmful associations with plants or to interactions studies combining multiple organisms (beneficial or harmful) on the same host.

Acknowledgements This work was supported by the Direction Générale de l'Agriculture of the Walloon Region under contract number D31-1149, entitled «Valorisation de la microflore bénéfique des sols pour le contrôle de la flore pathogène des productions de pomme de terre comme alternative à l'utilisation des pesticides». The authors are grateful to Pascale Massart, Phillipe Charue and Olivier Devroye for their technical assistance in the maintenance and propagation of the fungal strains *T. harzianum* and *R. solani* and GINCO for providing the AM fungus.

V. CHAPTER 2

Fast track *in vitro* mycorrhization of potato plantlets allows studies on gene expression dynamics

Adapted from **Mycorrhiza (2010) 20: 201-207**

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1. Preface

As demonstrated in the first chapter, the HAM-P *in vitro* culture system is an adequate tool to study the fungal life cycle. However, the establishment of the AM symbiosis (*i.e.* development of extraradical mycelium network and high-level of AM colonization) is a process that generally takes several weeks as compared with the fungal development of *T. harzianum* and *R. solani*. This difference in timing of fungal growth may complicate the interaction study between these organisms. One reason for the slow development of the AM fungus is attributed to the time needed for the AM fungal spore to germinate, grow, contact and colonize the root. Thus, the development of a system allowing the fast mycorrhization of seedlings (*i.e.* within a few days) and rapid re-growth of AM mycelium from the colonize plants is highly desirable.

In soil, persistent hyphal networks are the major mean by which plants become colonized (Smith and Read, 2008). Based on this observation, Voets *et al.* (2009) developed a mycelium donor plant (MDP) system for the fast and homogenous colonization of *Medicago truncatula* seedlings. In **Chapter 2** we aimed to confirm the results obtained by Voets *et al.* (2009) with potato plantlets and validate the MDP *in vitro* culture system as a tool to study gene expression during the process of AM fungal establishment.

2. Summary

Root colonization by arbuscular mycorrhizal (AM) fungi is a dynamic process involving major changes in plant gene expression. Here, the expression of a phosphate transporter gene (*PT3*) and several defense genes, already known to be involved in the various stages of AM establishment, were monitored in the mycelium donor plant (MDP) *in vitro* culture system associating potato plantlets with an AM fungus. This system allows fast and homogenous mycorrhization of seedlings at their early stage of development by growing the plantlets in active mycelial networks, but has never been validated for gene expression analysis. Here, QRT-PCR analyses were conducted in parallel to pre- (1 day), early (2 and 3 days), and late (6, 9, and 15 days) stages of root colonization. We observed the induction of a plant gene marker of AM root colonization (*PT3*) at the late stage and the induction of *MAPK* and *PAL* genes at the early and late stages of root colonization. We also demonstrated the induction of *PR1* and *PR2* genes at pre- and late stages and of *GST1* and *Lox* genes at a late stage of root colonization. These results validated the MDP *in vitro* culture system as an optimal tool to study gene expression analysis during the AM fungi establishment. This system further opened the door to investigate gene networks associated with the plants–AM fungi symbiosis.

Keywords: gene expression, *Glomus* sp., *in vitro* system, mycelium network, *Solanum tuberosum*

3. Introduction

Arbuscular mycorrhizal (AM) symbiosis represents a unique association between the mycelium of a soil-borne fungus and more than 80% of higher plants. The fungus, an obligate biotroph belonging to the phylum Glomeromycota (Schuβler *et al.*, 2001), receives carbohydrates required to complete its life cycle from the host plant; in exchange, it provides the plant with nutrients, such as phosphate. The establishment of this successful mutualistic association develops from a complex and dynamic process involving major changes in fungal and plant gene expression (Franken *et al.*, 2007; Reinhardt, 2007). During this process, phosphate transporter genes (Liu *et al.*, 1998) as well as transient defense-response genes are activated in the host plant (Liu *et al.*, 2003).

In the last few years, *in vitro* cultivation systems associating excised root organs with AM fungi have been used to investigate gene expression (González-Guerrero *et al.*, 2005; Elfstrand *et al.*, 2005; Waschke *et al.*, 2006). These systems present several advantages, among which are the absence of undesirable microorganisms and the possibility to nondestructively monitor the development of the fungal colony. However, some limitations are also associated to these systems, materialized by the absence of photosynthetic tissues: a normal hormonal balance and physiological source-sink relationships (Fortin *et al.*, 2002). In addition, the roots used in these experiments are, most often, genetically transformed organs.

Recently, Voets *et al.* (2005) and Dupré de Boulois *et al.* (2006) developed two *in vitro* autotrophic culture systems associating, respectively, potato and *Medicago truncatula* to root organ culture (ROC) produced AM fungal spores. Mass production of spores (*i.e.*, approximately 12,000 in 22 weeks) was obtained with potato (Voets *et al.*, 2005), and the transport of C from the shoot of *M. truncatula* to the fungus (Voets *et al.*, 2008) and of P and C from a root-free labeled compartment to the shoot via the extraradical mycelium (Dupré de Boulois *et al.*, 2006) was demonstrated. However, with both systems, high-level colonization took several weeks, and nearly full-grown mycorrhizal plants were produced before sampling. This may hinder experiments on gene expression analysis in plantlets during the early stages of root colonization. It is obvious that any system allowing the fast and homogenous mycorrhization of seedlings within a few days is highly desirable.

In nature, the AM fungal mycelium growing from colonized roots represents an important source of inoculum for the colonization of neighboring plants due to the several hyphal apices ramifying from the colony (Friese and Allen, 1991). Recently, Voets *et al.* (2009) have developed a new *in vitro* mycorrhization system adapted to seedlings by using the symbiotic phase of the fungus as inoculum for fast and homogenous AM colonization. This mycelium donor plant (MDP) *in vitro* culture system was successfully applied to *M. truncatula* plants opening large perspectives to study various aspects of the AM fungi and AM symbiosis.

In this study, we validated the MDP *in vitro* culture system of Voets *et al.* (2009) for gene expression analysis. The expression of a phosphate transporter gene (*PT3*) and several defense genes, already well known to be involved in the various stages of AM establishment, were analyzed at six time points corresponding to the establishment of *Glomus* sp. MUCL 41833 in roots of potato plantlets by combining the quantitative real-time-polymerase chain reaction (QRT-PCR) analyses with the assessment of intraradical root colonization.

4. Materials and methods

4.1. Biological material

4.1.1. Propagation and maintenance of stock of potato plantlets

Potato plantlets propagated *in vitro* (*Solanum tuberosum* L., var. Bintje) were supplied by the Station de Haute Belgique in Libramont, Belgium. Plantlets were micropropagated every 5 weeks as described in Voets *et al.* (2005).

4.1.2. Culture, propagation, and maintenance of *Glomus* sp. MUCL 41833

A root organ culture of *Glomus* sp. MUCL 41833, formerly identified as *Glomus intraradices* Schenck and Smith and presently reclassified into a clade contains the recently described species *Glomus irregulare* Blaszk., Wubet, Renker and Buscot (Stockinger *et al.*, 2009) was supplied by GINCO (http://www.mbla.ucl.ac.be/ginco-bel). The spores were extracted by solubilization of the gellan gel (Doner and Becard, 1991) and approximately 100 were placed in the near vicinity of actively growing transformed carrot (*Daucus carota* L.) roots (approximately 70 mm in length) on Petri plates (90 mm in diameter) containing the modified Strullu–Romand (MSR) medium (Declerck *et al.*, 1998 modified from Strullu and Romand, 1986), solidified with 3 g l^{-1} Phytagel (Sigma-Aldrich, St. Louis, USA)(Cranenbrouck *et al.*, 2005*)*. The Petri plates were incubated for 3 months in the dark in an

inverted position at 27°C, and several thousand spores were produced during this period.

4.1.3. *Medicago truncatula* seed disinfection

Seeds of *M. truncatula* Gaertn. cv. Jemalong A 17 (SARDI, Australia) were surface-sterilized by immersion in sodium hypochlorite (8% active chloride) for 12 min, rinsed three times in deionized sterile water and germinated in groups of 25 on Petri plates (90 mm in diameter) filled with 35 ml MSR medium without sucrose or vitamins, and solidified with 3 g l^{-1} Phytagel (Sigma-Aldrich, St. Louis, USA). Petri plates were incubated at 27°C in the dark.

4.2. Experimental design

Micropropagated potato plantlets were plated on actively growing extraradical mycelium networks of AM fungi developing in the hyphal compartment (HC) of bi-compartmental Petri plates (for details, see Voets *et al.*, 2009). The time course of gene expression was analyzed during symbiotic establishment in potato roots.

Eight weeks after association, a dense extraradical mycelium bearing numerous spores developed in the HC. The length of mycelium was 6261 cm ± 2367 and the number of spores, 19385 ± 9710 (estimated following the method of Voets *et al.*, 2005).

Two new holes (± 2 mm diameter), separated by a 4.5 cm distance from each other, were then made in the base and the lid of the Petri plates at the side of the HC. One ten-day-old potato plantlet was inserted in each hole following the same methodology described by Voets *et al.* (2009) with their roots in direct contact with the extraradical mycelium. The Petri plates were then sealed carefully and incubated in a growth chamber under controlled conditions, that is, 22/18°C (day/night), 70% relative humidity, photoperiod of 16 h d^{-1}, and an average photosynthetic photon flux of 225 µmol m^{-2} s^{-1}. Identically, two micropropagated potato plantlets were placed in the HC of the control treatment (*i.e.*, noninoculated 4-day-old *M. truncatula* seedlings) and grown under the same conditions as described previously.

Twenty-four cultural systems were randomly divided into 6 groups of four replicates. Roots were harvested 1, 2, 3, 6, 9, and 15 days after contact (dac) with the extraradical mycelium network of *Glomus* sp. MUCL 41833. One control plant was also harvested each time. For each cultural system, the two potato plantlets from the HC were pooled to obtain sufficient material for analysis. Half of the material was then used to estimate intraradical root colonization while the other half was used for gene expression analysis.

4.3. Intraradical root colonization

The intraradical root colonization was estimated under a dissecting microscope (Olympus SZ40, Olympus Optical GmbH, Hamburg, Germany) at ×10 to ×40 magnification, according to McGonigle *et al.* (1990). Roots were cleared in 10% KOH at 50°C for 90 min, rinsed with distilled water, and stained with trypan blue 1% (Phillips and Hayman, 1970) at 50°C for 60 min. Percentages of root colonization were subjected to one-way analysis of variance (ANOVA). Tukey's Honest Significant Difference (HSD) was conducted to identify significant differences ($p \leq 0.05$) between the groups. Data analysis was performed with the SAS enterprise guide 4.1 (SAS Institute Inc., Cary, USA).

4.4. RNA extraction

Total RNA was extracted from 50 to 100 mg frozen material with Trizol® reagent (Invitrogen, Carlsbad, USA) with an additional chloroform purification step and then purified using the Purelink™ Micro-to-Midi total RNA purification system (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The total RNA was subsequently treated with the TURBO DNA-free™ kit (Ambion, Austin, USA) according to the manufacturer's instructions. Concentration and purity of total RNA were determined in a NanoDrop®-ND 1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, USA) using a 2 µl aliquot of the total

RNA solutions. RNA purity was estimated from the A260/A280 absorbance ratio.

4.5. Reverse transcription

Following total RNA extraction, reverse transcription (RT) of 500 ng of RNA was performed with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Montreal, Canada) in a volume of 20 µl with Oligo$(dT)_{18}$ primer at 55°C for 20 min according to the manufacturer's instructions. For each RNA sample, a reaction without RT was performed as a control for contamination by genomic DNA.

4.6. Primer design

Four reference genes: glyceraldehyde phosphate dehydrogenase (*GAPDH*), ubiquitin conjugating enzyme-like (*Ubc*), elongation factor 1-alpha (*EF1-α*), and beta-tubulin (*β-tub*) (Nicot *et al. 2005*) and seven genes: gluthatione-s-transferase 1 (*GST1*), lipoxygenase (*Lox*), MAP kinase (*MAPK*), Pathogenesis Related 1 (*PR1*), Pathogenesis Related 2 (*PR2*), phenylalanine ammonia lyase (*PAL*), and phosphate transporter 3 (*PT3*) were selected. Potato nucleotide sequences were obtained from the GenBank database. Eleven primer pairs were designed from these sequences (90–110-bp length, optimal temperature of annealing at 60°C, GC% between 40% and 60%) with the LightCycler Probe Design Software 2.0 (Roche, Montreal, Canada).

The forward and reverse primer sets and melting temperatures (in brackets) were as follows: 5′-CCAAGTAACCTCTTGCTAAATGC-3′ and 5′-CTGTCATATTCTCGTTCTCTAGG-3 for *MAPK* (79°C); 5′-GCTTTGCTTACTTATTATTGGCG-3′ and 5′-GGAAGCAGCCTTAGTAGCATT-3′ for *PT3* (82°C); for the nine other primer sets, see Gallou *et al.* (2009).

4.7. Quantitative real-time PCR

QRT-PCR analysis was performed using the LightCycler 2.0 (Roche, Montreal, Canada). A set of standard solutions prepared from RT products was included in each run. Reactions were prepared in capillaries using the following concentrations: 7 µl of PCR water, 4 µl of 5× LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Mix (Roche, Montreal, Canada), 2 µl of each forward and reverse primer (0.5 µM), and 5 µl of 1:10 diluted cDNA or standard solution as template (for LightCycler experimental run, see Gallou *et al.* 2009). In order to check PCR efficiency, standard curves (log of cDNA dilution versus C_p) using serial 10-fold dilution of cDNA were created for each pair of selected primers. To obtain good comparison and normalization, PCR efficiency should range between 80% and 115%. In this study, all the PCRs displayed efficiencies between 91% and 104%. For the mathematical model, it was necessary to determine the crossing point (Cp) for each transcript, defined as the point at which the fluorescence rises appreciably above the background fluorescence. The Fit point method was performed in the LightCycler software 4.1

at which Cp was measured at a constant fluorescence level. The combination of several reference genes smooths out normalization error due to the small variation in the expression of a single reference gene (Vandesompele *et al.*, 2002). We determined the best combination of reference genes for normalization of gene expression by using the geNorm software (http://medgen.ugent.be/~jvdesomp/geNorm). The most stable genes expressed in the potato roots during symbiotic establishment with *Glomus* sp. MUCL 41833 were *β-tub* and *EF1-α*. The pairwise variation demonstrated that three reference genes (*i.e.*, *β-tub, EF1-α*, and *GAPDH*) were sufficient to normalize gene expression in potato plantlets grown in the extraradical mycelium network of *Glomus* sp. MUCL 41833 (data not shown). Normalization was achieved using the geometric means of the three reference genes, *i.e.*, *β-tub, EF1-α*, and *GAPDH*. The data were analyzed statistically with software REST 2008 (relative expression software tool; Pfaffl *et al.*, 2002; http://rerst.gene-quantification.info/). Significance values were set at P value ≤ 0.05. P(H1): the probability of alternate hypothesis that differentiates between sample and control groups is due only to chance.

5. Results

5.1. Time course of root colonization of potato plantlets grown in the extraradical mycelium network of *Glomus* sp. MUCL 41833

The intraradical root colonization of potato plantlets was assessed 1, 2, 3, 6, 9, and 15 dac of the root system with the AM fungal extraradical mycelium network (Table 3). No AM root colonization was observed 1 dac with the extraradical mycelium network. The first traces of AM fungal root colonization were detected 2 dac (1.9 ± 3%) and increased slightly (9.9 ± 7%) 3 dac. However, no significant differences were observed between the values recorded at 1, 2, and 3 dac. Neither arbuscules nor vesicles were observed at 1, 2, and 3 dac. Six dac, the AM root colonization increased markedly (24 ± 24%), and the first arbuscules and vesicles were observed. The percentages of arbuscules and vesicles observed 6 dac were 3.9 and 0.3, respectively. The AM root colonization estimated 9 dac was 48.2 ± 14% and remained almost identical 15 dac (49.9 ± 8%). The values of AM root colonization were significantly higher, 9 and 15 dac as compared with 1, 2, and 3 dac.

Table 3 Intraradical colonization of potato roots plated on actively growing extraradical mycelium networks of *Glomus* sp. MUCL 41833

		Intraradical root colonization during the time course[a]					
		1	2	3	6	9	15
Colonization (%)	Total	0 (±0) a	1.9 (±3) a	9.9 (±7) a	24 (±24) ab	48.2 (±14) b	49.9 (±8) b
	Arbuscules	0	0	0	3.9	13.6	8.2
	Vesicles	0	0	0	0.3	3.5	5.7

Values of colonization followed by different letters differ significantly at $p \leq 0.05$ (one-way ANOVA and Tukey's HSD). The standard deviations is shown in brackets (biological replicates = 4)

[a]Days after contact of potato plantlets with the network of *Glomus* sp. MUCL 41833

The 6 dac values recorded were intermediate between those obtained 1, 2, 3 dac and 9 and 15 dac. At 9 dac, the percentages of arbuscules and vesicles were 13.6 and 3.5, respectively. At 15 dac, the percentages of arbuscules and vesicles were 8.2 and 5.7, respectively.

5.2. Expression of marker gene of AM root colonization of potato plantlets grown in the extraradical mycelium network of *Glomus* sp. MUCL 41833

The relative expression ratio of *PT3* genes in potato roots was assessed 1, 2, 3, 6, 9, and 15 dac with the extraradical mycelium network (Table 4). We observed an induction of *PT3* gene 9 and 15 dac with a maximum of 15 dac [65.30 dac (19.90–191.08 dac)].

5.3. Expression of defense response genes in potato plantlets grown in the extraradical mycelium network of *Glomus* sp. MUCL 41833

The relative expression ratio of the defense response genes *GST1*, *Lox*, *MAPK*, *PAL*, *PR1*, and *PR2* in potato roots was assessed 1, 2, 3, 6, 9, and 15 dac with the extraradical mycelium network (Table 4). For the *GST1* gene, the induction of 6, 9, and 15 dac was detected with a maximum of 15 dac [5.68 dac (4.20–8.76 dac)]. For the *Lox* gene, the induction of 9 and 15 dac was detected with a maximum of 15 dac [6.12 (2.73–10.13)].

Table 4 Relative expression ratio of seven genes (gluthatione-S-transferase 1 (*GST1*), lipoxygenase (*Lox*), MAP kinase (*MAPK*), Pathogenesis Related 1 (*PR1*), Pathogenesis Related 2 (*PR2*), phenylalanine ammonia lyase (*PAL*), and phosphate transporter 3 (*PT3*)) in potato roots plated on actively growing extraradical mycelium networks of *Glomus* sp. MUCL 41833normalized by the geometric mean of the three reference genes (*i.e.*, *β-tub*, *EF1-α*, and *GAPDH*)

	Relative expression ratio during the time course[a]					
Gene name	1	2	3	6	9	15
PT3	1.86 [0.44–9.71]	0.24 [0.08–0.94]	1.81 [1.05–2.56]	0.28 [0.05–4.00]	*5.51 [2.58–16.02]*	*65.30 [19.90–191.08]*
GST1	1.40 [1.01–2.14]	1.41 [0.93–2.70]	0.30 [0.19–0.46]	*2.72 [2.39–3.10]*	*4.48 [2.93–7.47*]	*5.68 [4.20–8.76]*
Lox	1.00 [0.40–3.71]	0.63 [0.10–2.08]	0.62 [0.47–0.84]	1.80 [1.02–4.51]	*3.38 [1.26–7.60*]	*6.12 [2.73–10.13]*
MAPK	0.64 [0.17–2.09]	*5.74 [1.75–16.78]*	0.42 [0.32–0.56]	*2.64 [1.70–4.01]*	*2.01 [1.30–3.95]*	*8.12 [3.92–16.60]*
PAL	1.95 [0.83–5.11]	*7.82 [3.22–21.04]*	3.88 [1.52–14.37]	*12.10 [5.32–30.22]*	*3.06 [1.26–11.25*]	*28.55 [11.40–60.85]*
PR1	*2.50 [1.60–4.79]*	0.15 [0.05–0.69]	1.05 [0.70–1.63]	1.21 [0.55–2.31]	*7.35 [1.40–57.06]*	*105.31 [60.33–229.85]*
PR2	*3.94 [1.57–10.30]*	1.63 [0.67–3.47]	1.80 [0.98–3.27]	*13.39 [6.07–29.52]*	*10.51 [1.35–194.15]*	*159.94 [85.56–330.10]*

Data were analyzed statistically by the software REST 2008 (relative expression software tool; Pfaffl *et al.* 2002; http://rerst.gene-quantification.info/). Upregulated genes (*in italics*) with significance values were set at P value ≤ 0.05 (P(H1): the probability of alternate hypothesis that differentiates between sample and control groups is due only to chance). The standard error range is shown in brackets (biological replicates = 4).

[a]Days after contact of potato plantlets with the extraradical mycelium network of *Glomus* sp. MUCL 41833

The *MAPK* and *PAL* genes were induced 2 dac, and an induction was noted 6, 9, and 15 dac with a maximum of 15 dac [8.12 dac (3.92–16.60 dac)] for *MAPK* and [28.55 (11.40 – 60.85)] for *PAL*. For the two Pathogen Related genes (*i.e.*, *PR1* and *PR2*), an induction of 1 dac was detected, and respectively 9 and 15 dac with a maximum of 15 dac [105.31 dac (60.33 – 229.85 dac)] for *PR1*, and 6, 9, and 15 dac with a maximum of 15 dac [159.94 (85.56 – 330.10)] for *PR2*.

6. Discussion

Root organ cultures have been considered as suitable systems to investigate various aspects of the AM fungi and AM symbiosis (Fortin *et al.,* 2002). Recently, autotrophic nontransformed plants have been successfully cultured *in vitro* in association with AM fungi (Voets *et al.*, 2005; Dupré de Boulois *et al.*, 2006), and a fast-track *in vitro* mycorrhization system (*i.e.*, the MDP *in vitro* culture system) was developed (Voets *et al.,* 2009). This system opens large perspectives for molecular studies but should be validated for gene expression analysis. Here, the expression of a phosphate transporter gene and six defense genes were monitored by QRT-PCR and paralleled with the multistep process of potato AM fungal root colonization. Gene expression change could be observed in prestage (before root colonization, 1 dac), early stage (hyphal root colonization before arbuscule and vesicle formation, 2 and 3 dac), and the late stage of root colonization (intense root colonization with arbuscules and vesicles formed, 6, 9, and 15 dac).

During the precolonization stage of the potato–*Glomus* sp. MUCL 41833 interaction (1 dac), we observed the induction of *PR1* and *PR2* genes while no induction was noted during the following 2 and 3 dac (early root colonization stage). This change in gene expression between pre- and early stages of AM fungi root colonization was reported earlier by Kapulnik *et al.* (1996), Ruíz-

Lozano *et al.* (1999), and Liu *et al.* (2003) and associated with a transient level of defense-gene expression.

During the early stage of the potato–*Glomus* sp. MUCL 41833 interaction (2 and 3 dac), we observed the induction of the *MAPK* gene (at 2 dac). This observation is consistent with the role of MAP kinases in the signaling of plant abiotic stress and pathogen defense (Nakagami *et al.*, 2005) and corroborates the findings of Deguchi *et al.* (2007). The early stage of root colonization was also characterized by the induction of *PAL* gene as reported by Deguchi *et al.* (2007).

During the late stage of the potato–*Glomus* sp. MUCL 41833 interaction (6, 9, and 15 dac), we observed an induction of *PR1* (9 dac) and *PR2* (6 dac) with a buildup in the level of induction, paralleled thereafter with increased root colonization. *PR1* gene is a PR protein with antifungal properties and an unknown microbial target (Antoniw *et al.*, 1980). This gene is known to respond to fungal infection in potato (Gallou *et al.*, 2009). *PR2* gene is a β-1,3-glucanase induced, among others, in *M. truncatula* roots colonized by *G. intraradices* and *G. mosseae* during the late stage of root colonization (Hohnjec *et al.*, 2005).

We noted the induction of a *Lox* gene (*i.e.*, the first enzyme in the biosynthesis pathway of JA gene) 9 dac. This is in agreement with earlier results showing that, in roots of mycorrhizal plants, the levels of JA were higher as compared with non mycorrhizal controls

(Meixner *et al.*, 2005; Stumpe *et al.*, 2005; Hause *et al.*, 2007). We also observed a significant induction of *PAL* gene 6, 9, and 15 dac with increased percentages of arbuscules and vesicles in roots. Harrison and Dixon (1994) have demonstrated that transcripts encoding enzymes of the isoflavone biosynthetic pathway, such as *PAL* and chalcone synthase, are induced specifically in cells containing arbuscules.

Glutathione-s-transferase transcripts have been found to accumulate in roots containing arbuscules (Wulf *et al.*, 2003; Brechenmacher *et al.*, 2004). In our study, we observed the induction of the *GST1* gene in the potato roots 6, 9, and 15 dac in parallel with the formation of the first arbuscules and vesicles. We also observed the induction of an *MAPK* gene in the late stages of root colonization, as reported by Weidmann *et al.* (2004) and Grunwald *et al.* (2004) in *M. truncatula* roots colonized by *G. mosseae.*

During the late stage of the potato–*Glomus* sp. MUCL 41833 interaction (6, 9, and 15 dac), we observed the induction of the phosphate transporter gene *PT3* (9 dac) with a buildup in the level of induction, 15 dac, paralleled with an increase in root colonization. Rausch *et al.* (2001) have identified the phosphate transporter gene *StPT3* in potato and localized *PT3* induction specifically in arbuscule-containing cells.

During this study on gene expression analysis at the different stages of AM establishment with the MDP *in vitro* culture system, we observed the induction of *PT3* gene at the late stage of potato–*Glomus* sp. MUCL 41833 interaction. Two PR genes (*PR1* and *PR2*) were induced prior to root colonization with a transient expression at 2, 3, and 6 dac (for *PR1*) followed by a continuous buildup in the level of induction. We finally demonstrated the induction of *GST1*, *Lox*, *MAPK*, and *PAL* genes at different stages of potato–*Glomus* sp. MUCL 41833 interaction. The result obtained for the *PT3* gene (a plant gene marker of AM root colonization) demonstrated that the potato–*Glomus* sp. MUCL 41833 association was successfully established and the mutualistic exchange between the two partners was effective. The induction of defense genes, well known to be involved at different stages of the AM symbiosis, demonstrated that the potato–*Glomus* sp. MUCL 41833 establishment in the MDP *in vitro* culture system was suitable to study major changes in plant gene expression and corroborated previous results of *in vivo* studies. Our study opened new avenues to investigate the molecular events or gene networks associated with the plants–AM fungi symbiosis by making it possible to synchronize the development of AM fungi in the roots of plants grown in an established non-perturbed mycelium network under rigorous *in vitro* culture conditions.

Acknowledgements This research was sponsored by the Direction Générale de l'Agriculture de la Région wallonne under contract number D31-1149, entitled « Valorisation de la microflore bénéfique

des sols pour le contrôle de la flore pathogène des productions de pomme de terre comme alternative à l'utilisation des pesticides ». S. C. gratefully acknowledges the financial contribution of the Belgian Federal Science Policy Office (contract BCCM C3/10/003).

VI. CHAPTER 3

Mycoparasitism of arbuscular mycorrhizal fungi: a pathway for the entrance of saprophytic fungi into roots

FEMS Microbiology Ecology (2010) 73:312-322

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1. Preface

Several authors have demonstrated parasitism of AM fungal structure (spores and extraradical hyphae) by *Trichoderma* sp. (Lee and Koske, 1994; Rousseau *et al.*, 1996). The cytoplasmic continuity between extra- and intraradical mycelium may present a pathway for invasion of mycoparasites. However, no studies have addressed the possible parasitism of the mycelium within the roots.

To evaluate the impact of *T. harzianum* on the AM fungi, we developed two *in vitro* culture systems (see **Chapters 1** and **2**). With the MDP *in vitro* culture system we achieved the fast and homogenous colonization of potato plantlets. After transfer onto fresh medium in the HAM-P *in vitro* culture system, the mycorrhized plantlets were able to growth and reproduce the fungal life cycle. Based on the combination of both systems, we aimed to study in **Chapter 3** whether the mycoparasitism of *T. harzianum* is observed on the extra- and intra-radical mycelium of *Glomus* sp. and whether it may represent a pathway for the entrance of the saprotrophic fungi into the roots.

2. Summary

Within the rhizosphere, arbuscular mycorrhizal (AM) fungi interact with a cohort of microorganisms, among which is the biological control agent, *Trichoderma* spp. This fungus parasitizes a wide range of phytopathogenic fungi, a phenomenon also reported in the extraradical mycelium (ERM) of AM fungi. Here, we question whether the mycoparasitism of the ERM could be extended to the intraradical mycelium (IRM), thus representing a pathway for the entry of *Trichoderma harzianum* within the root. Microcosm experiments allowing interactions between *Glomus* sp. MUCL 41833 placed in a clade that contains the recently described species *Glomus irregulare* and *T. harzianum* were set up under *in vitro* autotrophic culture conditions using potato as a host. A microscope camera-imaging system, coupled with succinate dehydrogenase staining, was used to assess the mycoparasitism in the ERM and IRM. *Trichoderma harzianum* colonized the ERM of the AM fungus and spread into the IRM, before exiting into the root cells. Intrahyphal growth of *T. harzianum* caused protoplasm degradation, decreasing the ERM and IRM viability. ERM of the AM fungus represented a pathway for the entry of *T. harzianum* into the roots of potato. It further sets off the debate on the susceptibility of the AM fungi of being infected by microorganisms from the rhizosphere.

Keywords: arbuscular mycorrhizal fungi, *Trichoderma harzianum*, mycoparasitism, *in vitro* culture, succinate dehydrogenase.

3. Introduction

The use of beneficial microorganisms has become a reliable alternative to reduce the application of pesticides in modern agriculture (Brimmer and Boland, 2003). Several studies have demonstrated the beneficial effects of plant growth-promoting microorganisms [*e.g.* arbuscular mycorrhizal (AM) fungi] and biological control agents (*e.g. Trichoderma* spp.) to improve plant health and productivity (Harman *et al.*, 2004; Whipps, 2004; Avis *et al.*, 2008) and soil quality (Schloter *et al.*, 2003; Rillig and Mummey, 2006).

AM fungi are ubiquitous soil microorganisms that colonize the roots of nearly 80% of terrestrial plants (Smith and Read, 2008). These fungi provide the plants with water and nutrients (*i.e.* in particular, phosphorus) in exchange for carbon (Smith and Read, 2008). They promote plant growth and productivity and have been shown to induce plant-defence responses against pathogens (reviewed in Conrath *et al.*, 2006). The species within the *Trichoderma* genus are free-living microorganisms, frequently isolated from soil and plant roots (Harman *et al.*, 2004). They establish a beneficial association with plants (Harman *et al.*, 2004; Vinale *et al.*, 2007; Avis *et al.*, 2008) and are able to colonize the root surface and induce localized or systemic plant defence responses (Yedidia *et al.*, 1999). Fungal antagonism via competition for nutrient and space, antibiosis production and fungal parasitism have been reported extensively in

the literature with these microorganisms (reviewed in Harman *et al.*, 2004).

Both microorganisms (*i.e.* AM fungi and *Trichoderma* spp.) have been used independently to improve plant growth and/or to control root pathogens (Harman *et al.*, 2004; Whipps, 2004). Several studies have also considered the use of both microorganisms as mixed inoculants (Calvet *et al.*, 1992; Siddiqui and Mahmood, 1996; Vázquez *et al.*, 2000). Even though positive effects have been reported on plant growth and health (Calvet *et al.*, 1993; Linderman, 1994; Datnoff *et al.*, 1995; Chandanie *et al.*, 2005, 2009), a number of studies also reported negative effects on the AM fungal root colonization (Calvet *et al.*, 1993; Green *et al.*, 1999; Martinez *et al.*, 2004) and a concomitant reduction in plant performance (*i.e.* shoot and root dry weights) (McAllister *et al.*, 1994a, b; Martinez *et al.*, 2004). One hypothesis that could explain these negative effects is the antagonistic non target action of *Trichoderma* spp. via mycoparasitism on the development of AM fungi (Rousseau *et al.*, 1996; Harman *et al.*, 2004; Vinale *et al.*, 2007). The mycoparasitism describes a relationship in which one microorganism obtains nutrients from another, affecting host viability (Jeffries, 1995). Rousseau *et al.* (1996) observed, under *in vitro* culture conditions, that *Trichoderma harzianum* T-203 (redescribed as *Trichoderma asperellum*; Kullnig *et al.*, 2001) penetrated the thick host wall (i.e spores and hyphae) of AM fungi through local hydrolysis of the wall polymers and colonized all extraradical structures. However, such observations were not

reported in the intraradical phase of the AM symbiosis (*i.e.* developing into roots), even though it could be suspected because the extraradical and intraradical mycelia (ERM and IRM) are coenocytically connected.

We hypothesized that the penetration of *T. harzianum* into the ERM of *Glomus* sp. MUCL 41833 associated with *Solanum tuberosum* could be extended into the IRM, and could affect the viability and development of both phases of the AM fungi. In order to demonstrate the parasitism in the extraradical and intraradical phases of this AM fungus, we used the autotrophic *in vitro* culture system developed by Voets *et al.* (2005, 2009). Direct microscopic observations, coupled with staining and enzymatic analysis [*i.e.* succinate dehydrogenase activity (SDH)], were used to (i) follow the interaction between *T. harzianum* and the AM fungus, (ii) quantify the development of both microorganisms and (iii) evaluate the cytological damage to both phases of the AM fungus.

4. Materials and methods

4.1. Plant material propagation and maintenance

4.1.1. Potato plantlets stock

In vitro propagated potato plantlets (*S. tuberosum* L., var. Bintje) were supplied by the Station de Haute Belgique in Libramont, Belgium. Plantlets were micropropagated every 5–6 weeks by placing nodal cuttings in sterile culture boxes (90 × 60 × 50 mm, 20 cuttings per box) containing 50 ml of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) following the methodology described by Voets *et al.* (2005). Plantlets were kept in a growth chamber at 22/18 °C (day/night) and illuminated for 16 h day^{-1} under a photosynthetic photon flux of 300 µmol m^{-2} s^{-1}.

4.1.2. Disinfection of *Medicago* seeds

Seeds of *Medicago truncatula* L., cv Jemalong strain A 17 (SARDI, Australia) were surface-sterilized by immersion in a solution of sodium hypochlorite (8% active chloride) according to the protocol described by Dupré de Boulois *et al.* (2006). Seeds were germinated in Petri plates (90 mm diameter) containing 40 ml of the modified Strullu–Romand (MSR) medium (Declerck *et al.* (1998) modified from Strullu and Romand, 1986) lacking sucrose and vitamins and solidified with 3 g l^{-1} Phytagel™ (Sigma-Aldrich Inc., St. Louis) and adjusted to pH 5.5 before sterilization (121°C for 15 min). Twenty-

five seeds were placed on the surface of each Petri plate. The Petri plates were incubated in the dark at 27°C. Seeds germinated within 1–2 days and were ready to use after 4 days.

4.2. Fungal strains and culture conditions

4.2.1. AM fungal culture

The AM fungus used was *Glomus* sp. MUCL 41833, formerly identified as *Glomus intraradices* Schenck and Smith and presently reclassified into a clade that contains the recently described species *Glomus irregulare* Błaszk., Wubet, Renker and Buscot (Stockinger *et al.*, 2009). *In vitro* root organ cultures were purchased from the Glomeromycota *in vitro* collection (GINCO – http://www.mbla.ucl.ac.be/ginco-bel). Cultures were provided in Petri plates (90 mm diameter) on the MSR medium supplemented with 10 g l^{-1} sucrose, adjusted to pH 5.5 before sterilization and solidified with 3 g l^{-1} Phytagel™ (Sigma-Aldrich Inc.) (Cranenbrouck *et al.*, 2005). The Petri plates were incubated for 3 months in the dark in an inverted position at 27°C. Several thousand spores and an extensive mycelium were produced within this period.

4.2.2. *Trichoderma harzianum* culture

A culture of *Trichoderma harzianum* Rifai (MUCL 29707) was supplied by the Mycothèque de l'Université catholique de Louvain (MUCL) (http://bccm.belspo.be/about/mucl.php) on potato dextrose

agar (PDA) (Scharlau Chemie S.A., Barcelona, Spain). The strain was chosen because of its inability to penetrate the roots of *S. tuberosum* (Gallou *et al.*, 2009; De Jaeger *et al.*, 2010b). A plug of gel containing several conidia and mycelium was placed in a sterile glass tube (1.5 ml) filled with 0.4 mL of 1% sterile-distilled water–peptone (Duchefa, the Netherlands). The plug was swirled in the sterile-distilled water–peptone using a vortex mixer for 15 s and the suspension was serially diluted to 10^{-2}. From the 10^{-2} dilution, 50 µl was spread on PDA. The Petri plates were incubated in the dark at 25°C for 4 days and subsequently maintained at 4°C until needed.

4.3. *In vitro* mycorrhization of potato plantlets and co-inoculation with *T. harzianum*

A mycelium donor plant (MDP) *in vitro* culture system was set up for the fast and homogenous mycorrhization of potato plantlets following the methodology described by Voets *et al.* (2009). The MDP *in vitro* culture system consisted of bi-compartmented Petri plates in which 4-day-old *M. truncatula* seedlings were transferred to the root compartment and inoculated with approximately 100 spores of *Glomus* sp. MUCL 41833, extracted from the AM fungal culture following solubilization of the MSR medium (Doner and Bécard, 1991). The ERM, spreading in the hyphal compartment (HC), was used as an inoculum to colonize young potato plantlets in a short period of time. Thirty MDP *in vitro* culture systems were selected following the criteria described by Voets *et al.* (2009) (*i.e.* the number

of spores and length of hyphae in the HC were 19385 ± 9710 and 6261 ± 2367 cm, respectively). Two 10-day-old potato plantlets were introduced in the HC of each MDP *in vitro* culture system. The roots of the potato plantlets were in contact with the ERM network developing in the HC. The Petri plates were sealed carefully and incubated in a growth chamber set at 22/18°C (day/night) and illuminated for 16 h day^{-1} under a photosynthetic photon flux of 300 µmol m^{-2} s^{-1}. After 9 days of contact with the ERM network, the potato plantlets were removed from the MDP *in vitro* culture systems and transferred into the *in vitro* autotrophic culture system (Voets *et al.*, 2005), *i.e.* mono-compartmented Petri plates (90 mm diameter) containing a thin layer (15 ml) of MSR medium lacking sucrose and vitamins solidified with 3 g l^{-1} Phytagel™. Profuse hyphal proliferation growing out of the potato roots was observed within 2–3 days, showing that the plantlets had been colonized successfully by the AM fungus. After a period of 12 days, 24 plants were selected on the basis of the number of spores and hyphal length produced, estimated under a dissecting microscope (Olympus SZ40; Olympus Optical GmbH, Hamburg, Germany) at magnification × 6.7–40 following the methodology of Declerck *et al.* (1996a) and Giovannetti and Mosse (1980), respectively. The number of spores was 273 ± 181 and the length of hyphae was 533.8 ± 181.5 cm. The 24 Petri plates were then divided into two homogenous groups of 12 Petri plates having the same mean hyphal length and number of spores. One group (the AMF-control treatment) only contained *Glomus* sp. MUCL 41833

associated to the potato plantlets. The other group (the AMF-T treatment) was inoculated with *T. harzianum* (a 4 mm diameter plug of PDA medium containing several hyphae). The plug was carefully placed in the centre of the Petri plate in the vicinity of the roots and in direct contact with the ERM of the AM fungus. In addition, 12 Petri plates containing potato plantlets of the same age, but without AM fungi, were inoculated with *T. harzianum* following the same procedure as described above (T-control treatment). The 36 Petri plates were incubated horizontally in a growth chamber set at 22/18°C (day/night) and illuminated for 16 h day^{-1} under a photosynthetic photon flux of 300 μmol m^{-2} s^{-1}. One experimental unit consisted of a potato plantlet inoculated with either *Glomus* sp. MUCL 41833 (AMF-control treatment) or *T. harzianum* (T-control treatment) or with both organisms (AMF-T treatment).

4.4. Non destructive microscopic observations

A study of hyphal interactions between *Glomus* sp. MUCL 41833 and *T. harzianum* was performed through direct microscopic observations, *i.e.* without disturbing the three-dimensional configuration of the mycelia. The interaction region between both fungi was observed 1, 2, 3, 4, 7 and 15 days after inoculation of *T. harzianum*. For each subsample, 150 hyphal interactions were assessed. Spore production by the AM fungus was assessed 7 and 15 days after co-inoculation in the AMF-T and AMF-control treatments. Colony growth rates of *T. harzianum* in the AMF-T and the T-control treatments were

determined by recording the radial development of the colony 1, 2, 3, 4, 7 and 15 days after inoculation of *T. harzianum*. Conidia production was assessed 15 days after inoculation by adding 10 ml of sterile water to the MSR medium. The Petri plates were subsequently shaken for 5 min and a suspension was sampled using a micropipette to prepare serial dilutions (up to 10^{-4}). Suspensions that were suitable to obtain quantifiable conidia production were plated on PDA medium (three Petri plates per dilution). After 24 h of incubation, colonies were counted as CFU/cm^{-2} of medium.

All the measurements were conducted under a dissecting microscope (magnification ×6.7–40) and a compound bright-field light microscope (magnification ×50–250). Images of the different stages of the fungal interactions were captured using a digital camera (Leica DFC320; Leica Microsystems Imaging Solutions Ltd, Cambridge, UK) coupled to a compound bright-field light microscope (Olympus BH-2; Olympus Optical GmbH) and displayed on a computer using an image manager software (LEICA IM50, version 4.0; Leica Microsystems Imaging Solutions Ltd).

4.5. Harvest and data collection

For each treatment, two groups of six Petri plates were randomly separated. One group was harvested 7 days after inoculation and the other group 15 days after inoculation. Mycelia of both fungi were carefully extracted following solubilization of the MSR medium

(Doner and Bécard, 1991) in order to assess the ERM viability and mycoparasitism. In parallel, the root systems were divided into two homogenous samples to estimate (1) the AM fungal root colonization and (2) the IRM viability and mycoparasitism.

Root colonization was assessed by root staining according to the protocol described by Phillips and Hayman (1970) and colonization was estimated under a bright-field light microscope at × 50–250 magnification, following the methodology of McGonigle *et al.* (1990). For each subsample, 150 root intersections were assessed.

In order to evaluate the mycoparasitism in the IRM, root samples (*i.e.* cleaned from extraradical hyphae and spores) were subjected to enzyme digestion following a modification of the protocol described by van Aarle *et al.* (2004) [*i.e.* enzyme digestion solution (0.2% cellulase from *T. viride*, 7.5 U mg^{-1}; Sigma, and 0.1% pectinase from *Rhizopus* sp. 451 U mg^{-1}; Sigma)]. The root cortex was separated from the central cylinder using needles and forceps under a stereo-microscope at × 6.7–40 magnification.

The viability of the hyphae in the ERM and IRM of the AM fungi was estimated using the SDH staining technique (Saito, 1995). Samples of both phases were incubated overnight at room temperature in the staining solution (Saito, 1995). Samples were washed with deionized water for 15 min. The IRM was counterstained by immersion in 0.01% (w/v) acid fuchsin/lactoglycerol for 15 min

(Vierheilig *et al.*, 2005), while the ERM was immersed in 0.1% (w/v) acid fuchsin/lactic acid (Olsson *et al.*, 2002) for 15 min. Samples were mounted on slides in 100% glycerol. The mycoparasitism observed in the stained AM fungal structures was visualized under an epifluorescence microscope (magnification × 50–250) equipped with an excitation wavelength of 570 nm and a 590-nm-long pass fluorescence emission filter. The SDH activity was counted using the magnified intersects method (McGonigle *et al.*, 1990). For each subsample, 150 hyphae intersections were assessed. The intraradical hyphae were classified into SDH-active and -inactive hyphae, corresponding to dark granular staining or without staining, respectively. The extraradical hyphae were identically classified into SDH-active and -inactive hyphae. In the SDH-inactive hyphae, a sub-classification into three categories was performed: protoplasm-filled hyphae, but without SDH activity (*i.e.* segment with protoplasm), septated hyphae (*i.e.* segments devoid of protoplasm and with septation) and empty hyphae (*i.e.* segments devoid of protoplasm and without septation).

4.6. Statistical analysis

Data analysis was performed using the statistical software SAS Enterprise Guide 4 (SAS Institute Inc., 2006). Data that were normally distributed and had homogeneous variances were subjected to an ANOVA. The Tukey honest significant difference test was used to identify the significant differences ($p \leq 0.05$).

5. Results

5.1. Fungal development in the plant system

The autotrophic *in vitro* culture system associating the potato plantlet with *Glomus* sp. MUCL 41833 (AMF-control) or *T. harzianum* (T-control) showed good development of both microorganisms and the differentiation of their classical structures. In the AMF-control treatment, spore, branched absorbing structures and runner hyphae ramifying into secondary and lower order hyphae were observed, while in the T-control treatment, highly branched hyaline hyphae bearing hundreds of conidiophores were observed. The same structures were observed in the AMF-T treatment, with intermingled and overlapped mycelia of both fungi. The hyphae of *Glomus* sp. MUCL 41833 and *T. harzianum* could easily be distinguished. The hyphae diameter of *T. harzianum* is smaller and contains septa in contrast to the coenocytic structure (*i.e.* absence of septa in living hyphae) of the AM mycelium.

5.2. Characterization of the interaction between *Glomus* sp. MUCL 41833 and *T. harzianum* in the extraradical phase

Twenty-four hours post inoculation with *T. harzianum*, the first contacts with the ERM of the AM fungus were observed. No tropism (*i.e.* hyphal avoidance) was identified between hyphae belonging to both fungi. The hyphae of *T. harzianum* grew alongside (Fig. 10a1) and/or coiled around the hyphae of the AM fungus (Fig. 10a2).

Shortly after contact, a hyphal swelling forming an 'appressorium-like' structure or a 'T-shaped branch' was observed at several places on the surface of the hyphae of the AM fungus. Penetration was often observed in the primary hyphae (*i.e.* runner hyphae), rarely in lower order branching hyphae and never in branched absorbing structures. *Trichoderma harzianum* hyphae perforated the hyphal wall of alive (*i.e.* hyphae with a bidirectional cytoplasmic flow; Fig. 10a1) and dead AM mycelium (*i.e.* hyphae devoid of cytoplasm content and/or septated), proliferated (Fig. 10b1) and branched in the AM hyphae (Fig. 10b2). Several penetration points were visualized on the same AM hyphae (Fig. 10a2), although no changes in their morphology (size, shape) were observed. Near the penetration zone (at ~80 µm distance), protoplasm retraction was observed. However, this phenomenon was never preceded by septa formation, but the formation of empty hyphal segments (*i.e.* devoid of protoplasm). The formation of septa was never observed in the close proximity to penetration points, but far beyond the zone of interaction between both fungi, and could not be strictly attributed to infection because septa were also observed in non infected hyphae. The hyphae of *T. harzianum* were able to perforate septa, and to grow through the dead sections of AM hyphae (Fig. 10b2). After 72 h of co-culture, the first 'T-shaped branch' hyphae were observed on the spore surface of the AM fungus (Fig. 10c1).

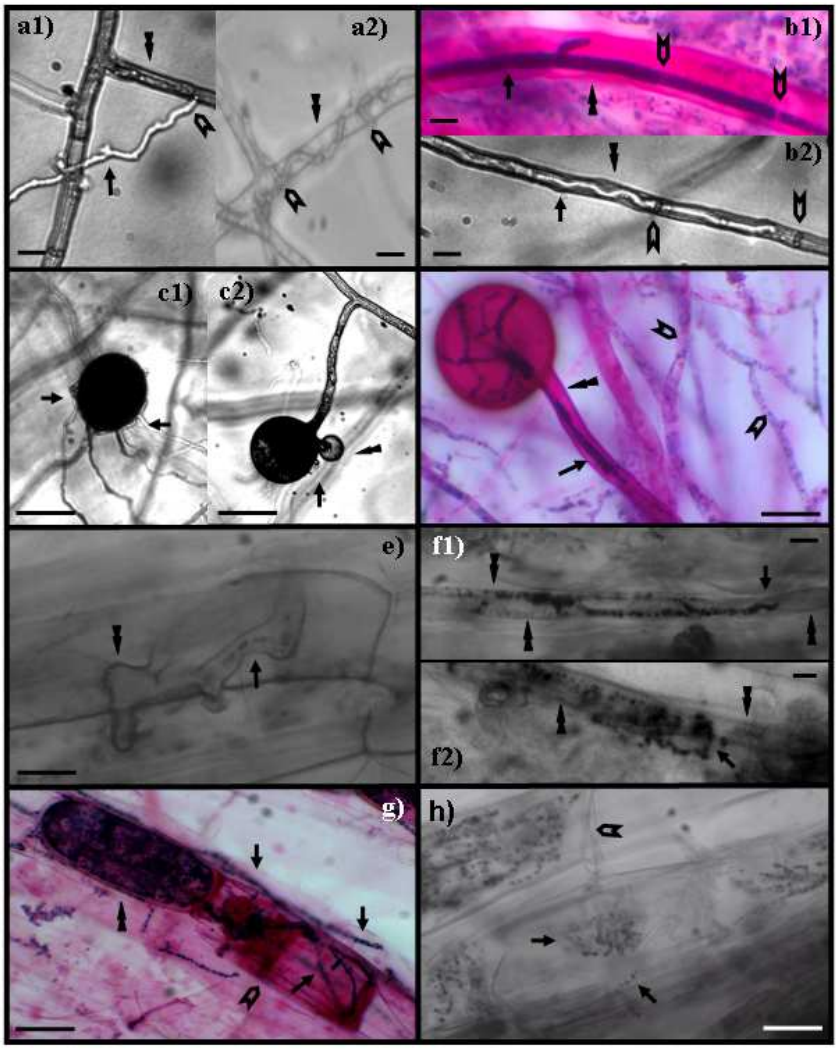

Figure 10 Microscope observations of the interaction between *Trichoderma harzianum* and the AM fungus *Glomus* sp. MUCL 41833 in association with *Solanum tuberosum* var. 'Bintje'. (a, b1, c) Cytological observations of theERM of the AM fungus. (b2, d) Microscope observation of the ERM of the AM fungus after SDH staining. (a) Recognition and contact between *T. harzianum* and the AM fungus, (a1) Contact (empty arrow) between *T. harzianum* (arrow) and the AM fungus hyphae (double arrowhead). Note that AM hyphae contained a cytoplasmic/protoplasmic flux at the contact point with *T. harzianum* (arrowhead) in contrast to the inferior hyphae section, which was septated (empty arrowhead), scale

bar=10 µm. (a2) *Trichoderma harzianum* coiling (empty arrows) around the hyphae of the AM fungus (double arrow), scale bar=10 µm. (b) Mycoparasitized hyphae of the AM fungus, (b1) hyphal invasion of *T. harzianum* (arrow) in the AM fungus hyphae (double arrowhead). Note that *T. harzianum* continued the infection, perforating the septa (empty arrow) and growing through the hyphal dead sections of AM fungal hyphae, scale bar=10 µm. (b2) *Trichoderma harzianum* colonization and ramification (arrow) in the main hyphae of the AM fungus (double arrow). Note the septum of *T. harzianum* (empty arrows) and absence of SDH activity in the hyphae of the AM fungus, scale bar=15 µm. (c) Spore mycoparasitism of the AM fungus, (c1) T-shaped branch hyphae of *T. harzianum* (arrows) over the spore surface of the AM fungus, scale bar=70 µm. (c2) Spore disruption and release of the spore contents (double arrowhead) caused by *T. harzianum* spore wall penetration (arrow), scale bar=70 µm. (d) Colonization of the subtending hyphae and penetration into the spore of the AM fungus by hyphae of *T. harzianum* (arrow). Note the absence of SDH activity in the subtending hyphae and the spore of the AM fungus (double arrow), scale bar=50 µm. (e–h) Microscope observations of the IRM of the AM fungus after SDH staining. (e) *Trichoderma harzianum* hyphae (arrow) within appressoria of the AM fungus (double arrow), scale bar=8 µm. (f) Intraradical mycoparasitized hyphae of the AM fungus, (f1) *T. harzianum* (arrow) colonization and ramification in IRM of the AM fungus (double arrow). Note that the hyphae of *T. harzianum* were positive to the SDH staining, suggesting a high activity, scale bar=10 µm. (f2) Exit of *T. harzianum* hyphae (arrow) from intraradical hypha of the AM fungus (double arrow) into the root environment, scale bar=10 µm. (g) SDH activity of the mycoparasitized vesicles. Note the differences in the SDH staining (*i.e.* dark granulation) between the uninfected lipid-filled vesicle (upper vesicle) (double arrow) and the mycoparasitized vesicle (lower vesicle) (empty arrow) by *T. harzianum* (arrows), scale bar=30 µm. (h) Root infection extra- (arrow) and intracellular (empty arrow) by peloton of hyphae of *T. harzianum*, scale bar=30 µm.

Single or multiple penetrations points occurred directly through the spore wall or via the subtending hyphae (Fig. 10d). Mycoparasitism was not linked with the spore age and size, because juvenile and mature spores were infected as well. Spore disruption and release of the spore contents were always associated with the spore wall penetration (Fig. 10c2).

No significant differences were observed in the total number of AM fungal spores produced in the AMF-T and the AMF-control treatments 7 and 15 days post-inoculation (Fig. 11a). After 7 days, the

number of spores produced ranged from 413 ± 67 in the AMF-T treatment to 412 ± 111 in the AMF-control treatment and after 15 days from 457 ± 97 in the AMF-T treatment to 492 ± 119 in the AMF-control treatment. Conversely, the number of damaged spores increased significantly in the AMF-T treatment as compared with the AMF-control treatment both after 7 and 15 days (Fig. 11a). After 7 days, the percentage of damaged spores in the AMF-T treatment was 7.4 ± 5.3%, while it was 0.4 ± 0.3% in the AMF-control treatment. After 15 days, the percentage of damaged spores in the AMF-T treatment was 9.0 ± 4.8%, while it was 0.6 ± 0.2% in the AMF-control treatment.

The enzyme histochemical observation showed that *T. harzianum* hyphae were positive to the SDH staining. Hyphae of *T. harzianum* showed a high density of dark granules characteristic of this particular staining. For the AM fungi, the dark granular staining was irregularly distributed within hyphae. A significant decrease in the viability of the AM fungus hyphae (SDH-active hyphae) was observed in the AMF-T treatment as compared with the AMF-control treatment (Fig. 11b). After 7 days, the SDH activity of the AM fungus hyphae was 44.0 ± 3.8% in the AMF-T treatment and 60.5 ± 4.0% in the AMF-control treatment, and after 15 days, the values were 26.8 ± 5.4% and 51.1 ± 3.4%, respectively. In addition, the percentage of mycoparasited hyphae of the AM fungus increased from 2.0 ± 0.9% to 3.9 ± 1.6% 7 and 15 days post *T. harzianum* inoculation, respectively (Fig. 11b).

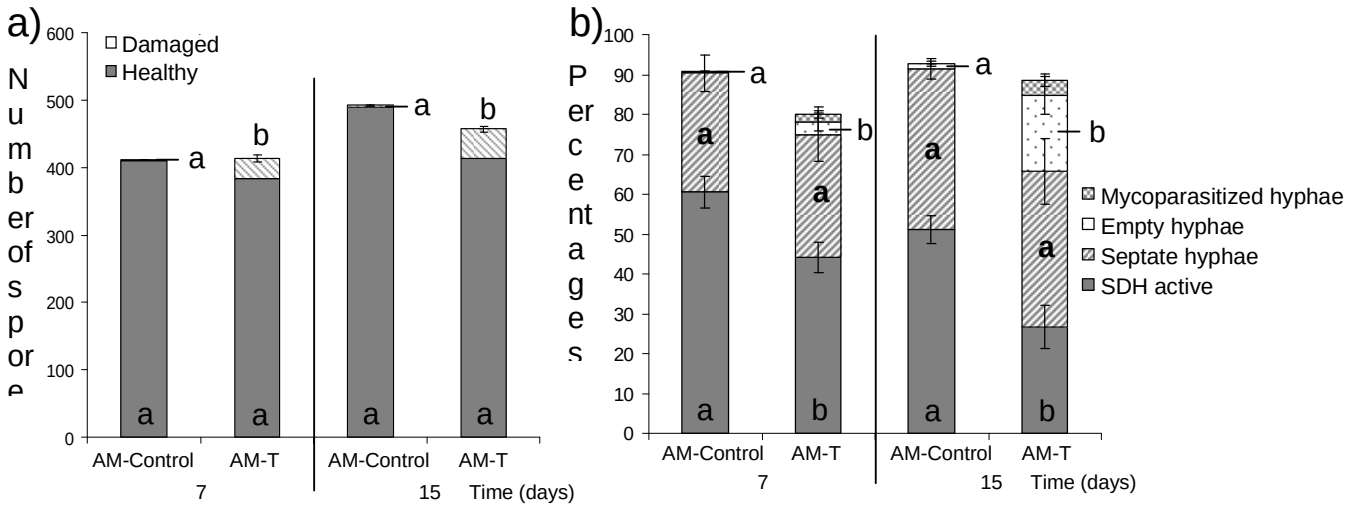

Figure 11 Impact of *Trichoderma harzianum* on the extraradical mycelium of the AM fungus *Glomus* sp. MUCL 41833 in association with *Solanum tuberosum* var. 'Bintje'. (a) Impact of *T. harzianum* on the spore production and percentages of damaged spores of the AM fungus, (b) Impact of *T. harzianum* on the percentages of SDH active hyphae, percentages of septate hyphae, percentages of empty hyphae and percentages of mycoparasitized hyphae of the AM fungus. For each time of observation (7 or 15 days) and parameter estimated, the values in the histograms followed by the same letter did not differ between the AMF-Control and AMF-T treatment (at $p \leq 0.05$, Tukey Honest test).

No significant differences were found in the percentage of septated hyphae between the AMF-T treatment and the AMF-control treatment. Values were 31.0 ± 6.7% in the AMF-T treatment and 30.0 ± 4.6% in the AMF-control treatment after 7 days and 38.8 ± 8.3% in the AMF-T treatment and 40.3 ± 2.6% in the AMF-control treatment after 15 days (Fig. 11b). On the other hand, the presence of *T. harzianum* significantly increased the percentage of AM hyphae devoid of protoplasm content (*i.e.* empty hyphae). After 7 days, the percentage ranged from 0.4 ± 0.8% in the AMF-control treatment to 3.0 ± 2.2% in the AMF-T treatment, and after 15 days, ranged from 1.2 ± 1.8% in the AMF-control treatment to 19.1 ± 4.8% in the AMF-T treatment.

The development of *T. harzianum* was not affected by the presence of the AM fungus. The growth rate percentage of *T. harzianum*, as indicated by the radius of its colony measured from the inoculation point (*i.e.* centre of the colony to the external edges), was the same in the AMF-T treatment as in the T-control treatment. The mean values were 0.8 mm at 24 h, 18 mm at 48 h, 31.5 mm at 72 h and 45 mm at 96 h post-inoculation. Four days after inoculation, the first conidiophores were observed in the centre of the colony on the surface of the medium. Gradual changes in the colour of the colony from white to green and finally greyish-green, following the production and maturation of the conidia, were observed. Contacts with roots were observed, but the direct and scrupulous observations

of roots under a bright-field microscope did not reveal any structure resembling appressorium or *T. harzianum* root penetration.

Conidial density of *T. harzianum* on the MSR medium of the AMF-T treatment was similar to that of the Th-control treatment. Fifteen days after *T. harzianum* inoculation, the number of conidia produced did not differ among the treatments. The values ranged from 545 728 ± 76 275 CFU cm^{-2} in the AMF-T treatment to 573 760 ± 65 834 CFU cm^{-2} in the T-control treatment.

5.3. Characterization of the interaction between *Glomus* sp. MUCL 41833 and *T. harzianum* in the intraradical phase

The direct observation under bright-field light microscope of the roots of *S. tuberosum* showed the presence of *T. harzianum* in the appressoria and in the intraradical fungal structures produced by *Glomus* sp. MUCL 41833. Seven days after *T. harzianum* inoculation, hyphae were observed within the appressoria of the AM fungus (Fig. 10e). *Trichoderma harzianum* was growing and branching following the hyphal branching architecture and structure of the AM fungus on the root surface (Fig. 10e). At this time, the observation of IRM revealed the presence of *T. harzianum* within intraradical hyphae of the AM fungus (Fig. 10f1). The intraradical AM parasitized hyphae were usually empty without any sign of viability (*i.e.* no SDH activity) and protoplasm content (Fig. 10f1). However, no perforation of the hyphal walls of AM hyphae was observed. Isolation of the infected

zone by septa formation was never observed. The presence of *T. harzianum* was observed neither in arbuscules nor in vesicles 7 days post-inoculation.

After 15 days, the presence of *T. harzianum* was observed inside the vesicles (Fig. 10g), but not in the arbuscules. The parasitized vesicles were usually empty, without SDH activity, in contrast to the uninfected lipid-filled vesicles (Fig. 10g). No perforation of walls, disruption of vesicles and release of lipid and organelle contents or changes in the morphology (*i.e.* size and shape) of the parasitized AM vesicles were observed. The microscopic observation of parasitized hyphae of the AM fungus showed the exit of *T. harzianum* (Fig. 10f2) directly through the hyphal wall colonizing the intercellular space and penetrating into the root cells (epidermis and cortical parenchyma) of *S. tuberosum* (Fig. 10h). The root infection by *T. harzianum* was always in patches, where peloton of hyphae were observed in root cells close (~1–10 mm) to the intraradical AM fungal structures (Fig. 10h). The root cells in intimate contact with *T. harzianum* did not show any signs of necrosis. No structures resembling conidiophores were observed in the roots during the entire experiment.

The enzyme histochemical observation showed a significant decrease of the intraradical hyphae viability of *Glomus* sp. MUCL 41833 (*i.e.* SDH active hyphae) in the presence of *T. harzianum* compared with the AMF-control treatment. After 7 days, the AM

fungus hyphae viability was 87.7 ± 4.4% in the AM-control treatment and 69.9 ± 4.1% in the AMF-T treatment, and after 15 days, the AM fungus hyphae viability was 77.8 ± 4.9% in the AMF-control treatment and 58.9 ± 6.1% in the AMF-T treatment.

No differences in total colonization, arbuscular and vesicular colonization were observed between treatment and control during the experimental period (Table 5). The total root colonization after 15 days ranged from 31.5 ± 7.2% in the AMF-control treatment to 28.5 ± 7.6% in the AMF-T treatment.

Table 5 Impact of *Trichoderma harzianum* on the percentage of root colonization (total, arbuscular and vesicular) of the AM fungus *Glomus* sp. MUCL 41833 associated with autotrophic potato plantlets (*Solanum tuberosum*).

Time (days)	7		15	
	AMF-Control	AMF-T	AMF-Control	AMF-T
Colonization total (%)	18.6 ± 5.1a	25.8 ± 7.7a	31.5 ± 7.2a	28.5 ± 7.6a
Arbuscular colonization (%)	3.2 ± 0.7a	6.8 ± 4.1a	5.7 ± 2.5a	8.5 ± 1.5 a
Vesicular colonization (%)	1.4 ± 0.6a	3.0 ± 0.6a	3.3 ± 1.5a	4.3 ± 1.7a

Values in the same row, followed by identical letters and within the same time (*i.e.* 7 or 15 days) did not differ significantly at $p \leq 0.05$ (Tukey test). Data represent the means of six replicates (mean±standard deviations).

Parasitized hyphae of the AM fungus represented <0.1% of the examined hyphae. It was 0.006 ± 0.004% and 0.012 ± 0.003% after 7 and 15 days, respectively.

The observation of the roots of the T-control treatment after Trypan blue staining and SDH, coupled with Acid fuschin staining, did not reveal the presence of *T. harzianum* inside the roots.

6. Discussion

This study demonstrated that the mycelium of the AM fungus *Glomus* sp. MUCL 41833 acted as a channel for the entry of *T. harzianum* into the roots of *S. tuberosum*. This phenomenon occurred through a multistep dynamic invasion process of the ERM (*i.e.* extraradical hyphae and spores) involving contact, surface attachment and penetration, and its subsequent extension into the IRM (*i.e.* intraradical hyphae and vesicles) before exiting in the root cells (Fig. 12). Intrahyphal growth of *T. harzianum* caused protoplasm degradation, decreasing the ERM and IRM viability.

Our observations (in the Th-control treatment) and two recent studies (Gallou *et al.*, 2009; De Jaeger *et al.*, 2010b) demonstrated that *T. harzianum* MUCL 29707 is unable to penetrate the roots of potato plantlets grown *in vitro*. Investigating plant-defence genes in potato plants grown in the presence of this fungus, Gallou *et al.* (2009) observed a slight induction of the phenylalanine ammonia lyase (*PAL*). *PAL* gene activation and high-level concentration of this enzyme within plant tissues has been strongly related to induced defence responses in plants against pathogens and/or saprotrophic fungi (Guo *et al.*, 2000; Yedidia *et al.*, 2003). The results obtained by Gallou *et al.* (2009) were therefore attributed to the absence of root cell penetration and colonization by the saprobe.

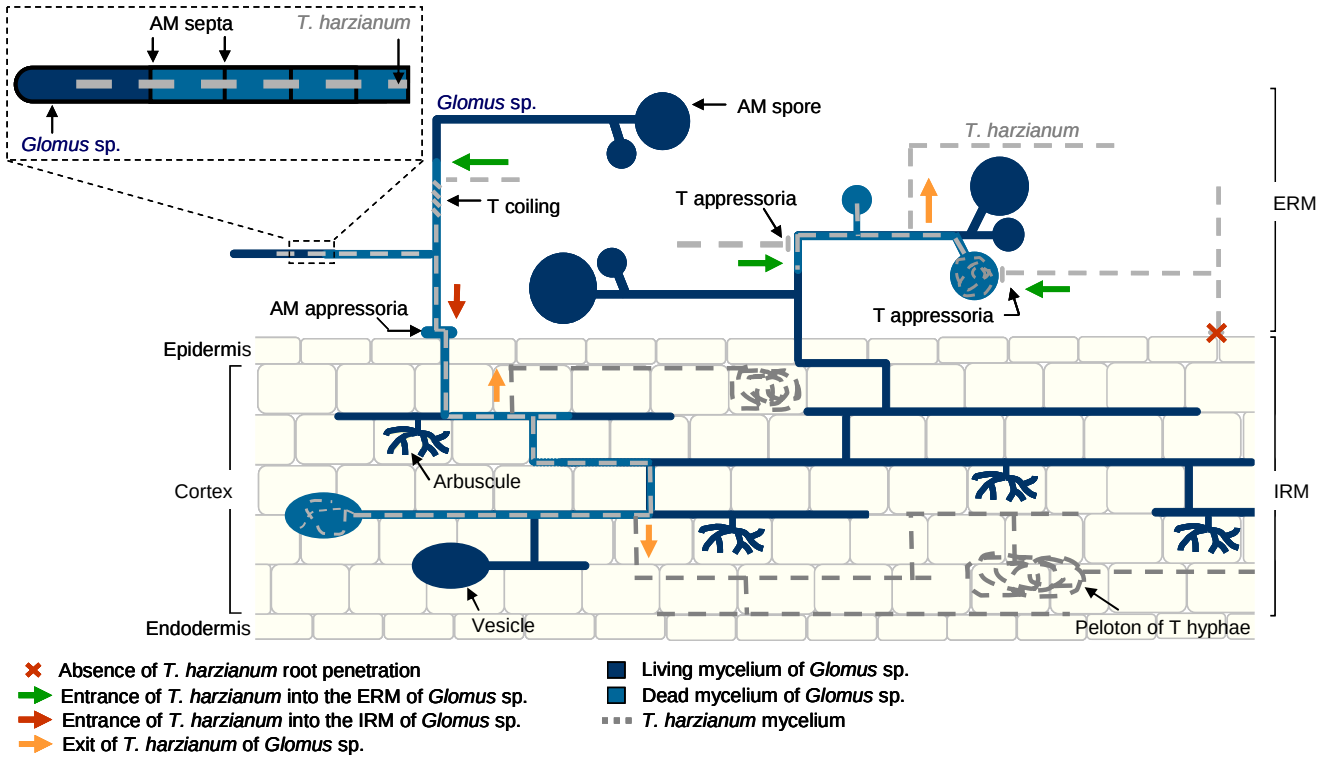

Figure 12 Caption opposite.

Figure 12 Schematic drawing of the entry of *Trichoderma harzianum* into *Solanum tuberosum* var. 'Bintje' root. *Trichoderma harzianum* growth alongside and/or coil around the hyphae of the AM fungus *Glomus* sp. MUCL 41833 before penetration of hyphae and/or spores. The mycoparasitism of the ERM of the AM fungus led to IRM invasion via the appressoria. Intra-hyphal growth of *T. harzianum* caused cytoplasm/protoplasm degradation, decreasing the ERM and IRM viability. *Trichoderma harzianum* perforated the septa to join the living section. *Trichoderma harzianum* exited the IRM to colonize the root in patches (*i.e.* peloton of hyphae).

In addition, neither appressorium formation nor root penetration and subsequent colonization were observed using scanning electron microscopy and a bright-field microscope (De Jaeger *et al.*, 2010b). It can be concluded that the presence of *T. harzianum* MUCL 29707 within the potato roots was exclusively related to its entry via the AM fungi *Glomus* sp. MUCL 41833. The coenocytic nature (*i.e.* the absence of septa in living hyphae) of the AM mycelium linked to the protoplasmic continuity between the extra- and the intraradical phase of AM fungi (Smith and Read, 2008) may aid this process and represent a unique pathway for the entry of *T. harzianum* into the roots.

Trichoderma species are free-living fungi that do not form obligate associations with plants (Harman *et al.*, 2004). They are mostly saprotrophic fungi in soil and dead plant material, but they can also be opportunistic plant root colonizers (Harman *et al.*, 2004). Some *Trichoderma* strains are able to colonize root surfaces and penetrate below the epidermis (Yedidia *et al.*, 1999; Harman *et al.*, 2004; Chacon *et al.*, 2007). So far, only the study of Evans *et al.* (2003) has reported the colonization of the vascular system of cocoa by these fungi (*i.e. T. harzianum, T. hamatum, T. virens, T. viride* and

four undescribed species). To date, there is no information on the mechanisms that govern *Trichoderma* spp. root colonization (Rincon *et al.*, 2009). One study has reported that *T. harzianum* develops penetration-specific morphological structures, such as appressoria and papillae (Chacon *et al.*, 2007). Once the fungus is inside the root, it branches out extensively to form highly branched haustoria without the disruption of root cells (Franken *et al.*, 2002). The absence of a detrimental effect on the plant, also observed under our experimental conditions, is not in contradiction with other studies (Harman *et al.*, 2004; Vinale *et al.*, 2007) because *Trichoderma* spp. is mostly used as a biocontrol.

Modification of *T. harzianum* growth and development was reported when the AM symbiosis was fully established (St Arnaud *et al.*, 1995; Green *et al.*, 1999). This modification could be directly caused by the AM fungi (*i.e.* hyphal exudates, competition for food and space) or indirectly by modification of the physiological and biochemical properties of the host plant following AM fungi establishment (*i.e.* modification of root exudation) (Avis *et al.*, 2008). In our experiment, no antagonistic effects or modifications of the growth of *T. harzianum* were observed in the presence of the already well-established AM fungal colony. This discrepancy may be attributed to several factors, among which are the experimental conditions as well as the plants and fungal species/strain used in the interaction.

Trichoderma harzianum was able to extend and ramify in the AM fungal hyphae without apparent modification in the hyphae structure. Despite being absent in living hyphae of Glomeromycota, septum formation is frequently observed in AM fungi as (1) a mechanism to restrict damage in the mycelium caused by physical stresses (de Souza and Declerck, 2003; de la Providencia *et al.*, 2005), (2) a strategy to survive in the absence of a suitable host (Logi *et al.*, 1998) and, more largely, (3) a mechanism associated with ageing or lytic events (Bago *et al.*, 1999). Under our experimental conditions, no physical barrier (*e.g.* septum formation to isolate infected segments) was observed close to the appressoria formed by *T. harzianum* on the hyphae of *Glomus* sp. MUCL 41833 and it is necessary to stress here that no significant differences were observed in the number of hyphae with septa between the AMF-control treatment and the AMF-T treatment. Hence, one may suppose that septation is a consequence of an ageing process rather than a response associated with a defence mechanism triggered by the presence of *T. harzianum*. Moreover, the high percentage of empty hyphae in the AM fungal colony suggested a rapid degradation of the hyphae content before septa formation in the living AM mycelium. Our results, combined with those obtained by Rousseau *et al.* (1996), suggested that *T. harzianum* invasion was so rapid and aggressive that AM fungi did not have enough time to trigger eventual defence systems, such as septum formation. However, this does not exclude that AM fungi may have developed defence mechanisms to counteract fungal invasion.

At present, little is known on the impact of *T. harzianum* on the growth and development of AM fungi. This might be because most studies were conducted under greenhouse and field conditions and have addressed the plant performance (Datnoff *et al.*, 1995; Siddiqui and Mahmood, 1996) rather than the interaction between both fungi. In our study, *T. harzianum* had no negative effect on the AM root colonization or sporulation during the experiment period (2 weeks). A decrease in root colonization by the AM fungi was reported by McAllister *et al.* (1994a) when *T. harzianum* was inoculated before or at the same time as the AM fungi. *Trichoderma harzianum* affected the AM root colonization by impacting the presymbiotic phase (McAllister *et al.*, 1994b). In contrast, when *T. harzianum* was inoculated after the establishment of the AM symbiosis, absence of a negative effect on AM root colonization was observed (McAllister *et al.*, 1994b; Green *et al.*, 1999; Vázquez *et al.*, 2000), conditions that were similar to those in our study. The absence of a negative impact of *T. harzianum* on extraradical mycelium growth observed in our study was also shown by Green *et al.* (1999) in a root-free soil experiment.

Although evidence of the mycoparasitism of the extraradical mycelium of AM fungi has been shown (Rousseau *et al.*, 1996), there have been very few studies that have demonstrated the relevance of this phenomenon. The present result showed clearly that *T. harzianum* extended from the ERM to the IRM and decreased the viability of the AM fungi *Glomus* sp. MUCL 41833. The low presence of *T. harzianum* into IRM could possibly be explained by a protection

effect of the root. In addition, it must be stressed that the access of *T. harzianum* to the IRM was exclusively via the extension of the mycoparasitism of the ERM and never via direct penetration of the root. The low percentage of mycoparasitism of the IRM suggested that the decrease in intraradical hyphae viability observed is due to the ERM–*T. harzianum* interaction rather than the mycoparasitism of the IRM. This is not in contrast to the results of McAllister *et al.* (1994a), who reported a decrease of the SDH activity as a consequence of AM fungus–microorganism interaction. A decrease in AM viability may affect the AM fungal growth and development in long-term periods, but may also modify the functioning of the AM symbiosis (*i.e.* transport of nutrient, etc.). It has been suggested that to compensate the negative effects caused by the mycoparasitism, AM fungi could modify the bidirectional transfer of nutrients, increasing the carbon transfer from plant to AM fungi and decreasing the soil-derived nutrient supply from the AM fungi to the plant, thus modifying the plant relationship from mutualism to 'parasitism' (Purin and Rillig, 2008). A change in mycorrhizal functioning could also explain the decrease of shoot and root dry plant weights (*i.e.* fitness and productivity) (McAllister *et al.*, 1994a; Klironomos, 2003; Lendzemo *et al.*, 2004; Martinez *et al.*, 2004).

In conclusion, mycoparasitism by *T. harzianum* was observed in all fungal structures of the ERM (*i.e.* in hyphae and spores), and for the first time, to our knowledge, within the IRM (*i.e.* in intraradical hyphae and vesicles) of the AM fungus *Glomus* sp. MUCL 41833.

This observation sets up the debate on the susceptibility of AM fungi of being infected by microorganisms (*i.e.* pathogens or biological control agents) from the rhizosphere and to act as a channel for root penetration. Further studies must be performed to evaluate the impact *T. harzianum* on the growth, functionality and survival of the AM fungus and to assess the influence of the dual inoculation on plant performance.

Acknowledgements This research was sponsored by the Direction générale opérationnelle de l'Agriculture, des Ressources naturelles et de l'Environnement du service public de wallonie under contract number D31-1193, entitled 'Valorisation de la microflore bénéfique des sols pour le contrôle de la flore pathogène des productions de pomme de terre comme alternative à l'utilisation des pesticides'. The authors thank Dr I. van Aarle, Dr H. Rouhier and Dr H. Dupré de Boulois for technical advice and helpful comments.

VII. CHAPTER 4

Trichoderma harzianum might impact phosphorus transport by arbuscular mycorrhizal fungi

Submitted to FEMS Microbiology Ecology

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1. Preface

In **Chapter 3**, we demonstrated that *T. harzianum* was able to feed on the intraradical and extraradical mycelium of AM fungi. The parasitism of *Glomus* sp. was followed by a rapid degradation of the AM cytoplasmic content affecting its viability. This process may possibly impact the capacity of the AM fungus to transport nutrients from the soil to the plant, an essential function attributed to this obligate symbiont. In **Chapter 4**, we aimed to evaluate the effect of *T. harzianum* on the transport of P by the AM fungus and parallel the observations with the SDH and ALP activities in the extraradical mycelium.

2. Summary

Trichoderma sp. is a biocontrol agent active against plant pathogens via mechanisms such as mycoparasitism. Recently it was demonstrated that *T. harzianum* was able to parasitize the mycelium of an arbuscular mycorrhizal (AM) fungus thus affecting its viability. Here, we question whether this mycoparasitism may reduce the capacity of *Glomus* sp. to transport phosphorus (^{33}P) to its host plant in an *in vitro* culture system. ^{33}P was measured in the plant and in the fungal mycelium in presence/absence of *T. harzianum*. The viability and metabolic activity of the extraradical mycelium was measured via succinate dehydrogenase and alkaline phosphatase staining. Our study demonstrated an increased uptake of ^{33}P by the AM fungus in presence of *T. harzianum* possibly related to a stress reaction caused by mycoparasitism. In addition, the disruption of AM extraradical hyphae in presence of *T. harzianum* affected the ^{33}P translocation within the AM fungal mycelium and consequently the transfer of ^{33}P to the host plant. The effects of *T. harzianum* on *Glomus* sp. may thus impact the growth and function of AM fungi, and also indirectly plant performance by influencing the source-sink relationship between the two partners of the symbiosis.

Key words: Arbuscular mycorrhizal fungi, *Trichoderma harzianum*, Phosphorus, Radio-labelling, SDH and ALP mycorrhizal activities, *in vitro.*

3. Introduction

Arbuscular mycorrhizal (AM) fungi and other beneficial soil-borne microorganisms such as *Trichoderma* spp. have been shown to improve plant productivity and health, and are thus of particular interest for sustainable agriculture (Whipps, 2004; Harman *et al.*, 2004; Avis *et al.*, 2008). Their combination has been reported in several studies but with contrasting results. Several studies have demonstrated a positive effect of the dual inoculation on plant performance in presence as well as in absence of plant pathogens (Datnoff *et al.*, 1995; Siddiqui and Mahmood, 1996; Chandanie *et al.*, 2009), while other reported a reduction in plant shoot and root dry weights (McAllister *et al.*, 1994a, b; Martinez *et al.*, 2004). One hypothesis that could explain the later is the antagonistic non-target action of *Trichoderma* spp. via mycoparasitism on the AM fungal mycelium (Lee and Koske, 1994; Rousseau *et al.*, 1996; De Jaeger *et al.*, 2010a).

Phosphorus is required by plants in relatively large amount but is often poorly available. Low solubility and slow diffusion of P in soil solution cause plants to become P deficient and to grow poorly (Smith and Read, 2008). To overcome this limitation, plants have developed several mechanisms of P acquisition, among which the association with AM fungi (Smith and Read, 2008). These microorganisms form symbiotic associations with nearly 80% of terrestrial plants (Smith and Read, 2008). This symbiosis is

characterized by a reciprocal exchange of nutrients (principally P) for carbon (Smith and Read, 2008). To increase plant P acquisition, the extraradical mycelium (ERM) of AM fungi extends into the soil, increasing significantly the absorptive area of roots (van der Heijden *et al.*, 2008). This indirect pathway of plant P acquisition (Smith *et al.* 2003) can be subdivided into three steps which are the uptake of P by the ERM, its translocation within the hyphae towards the intraradical structures of the AM fungus and its transfer from the fungal to the plant (Cooper and Tinker, 1978; Jakobsen *et al.*, 1992; Smith and Read, 2008).

The saprotrophic fungi from the genus *Trichoderma* are opportunistic, avirulent plant colonizers, which may act as parasites and antagonists (*i.e.* through competition for nutrient and space, antibiosis production) of many plant pathogens (Harman *et al.*, 2004). They have been shown to promote plant growth via increased nutrient uptake, and to protect them against biotic and abiotic stresses (reviewed in Harman *et al.*, 2004; Vinale *et al.*, 2007; Avis *et al.*, 2008). Mycoparasitism by *Trichoderma* spp. has been reported as a major mechanism to control plant fungal pathogens. This mechanism was also observed on AM fungi in several studies (Lee and Koske, 1994; Rousseau *et al.*, 1996; De Jaeger *et al.*, 2010a). Recently, De Jaeger *et al.* (2010a) demonstrated that *T. harzianum* was able to parasitize the ERM of an AM fungus but also the hyphae developing within the roots, thus affecting its viability. It is therefore possible that the AM functionality, including the capacity to transport nutrients and

carbon, may be affected, further resulting in a lower performance of both partners of the symbiosis. However, the impact of mycoparasitism on the capacity of AM fungi to transport P or to acquire C from their host plants remains unknown.

Here we investigated the impact of *T. harzianum* MUCL 29707 on the transport of P by the AM fungus *Glomus* sp. MUCL 41833 to its host plant, *Solanum tuberosum* L. var Bintje. We adapted a compartmented *in vitro* culture system (Dupré de Boulois *et al.*, 2009) allowing the AM fungal ERM to develop in a ^{33}P-labelled compartment from which the mycorrhizal host roots and the saprotrophic fungi were physically excluded. ^{33}P was measured in the plant (shoot and roots) and in the fungal mycelium in presence/absence of *T. harzianum*. Data were confronted to the viability and metabolic activity of the extraradical hyphae of the AM fungus measured via enzyme activities of the succinate dehydrogenase (SDH) and alkaline phosphatase (ALP), respectively (Olsson *et al.*, 2002).

4. Material and methods

4.1. Potato plantlets stock

In vitro micro-propagated potato plantlets (*Solanum tuberosum* L., var. Bintje) were supplied by the Station de Haute Belgique in Libramont, Belgium. Plantlets were subcultured every 5-6 weeks by placing nodal cuttings in sterile culture boxes (90 × 60 × 50 mm, 20 cuttings per box) containing 50 ml of Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) following the methodology described by Voets *et al.* (2005). Plantlets were kept in a growth chamber at 22/18°C (day/night) and illuminated for 16 h d^{-1} under a photosynthetic photon flux (PPF) of 300 µmol m^{-2} s^{-1}.

4.2. Disinfection of *Medicago* seeds

Seeds of *Medicago truncatula* L., cv Jemalong strain A 17 (SARDI, Australia) were surface-disinfected by immersion for 15 min in a solution of sodium hypochlorite (8% active chloride) according to Dupré de Boulois *et al.* (2006). Seeds were then placed for germination on Petri plates (90 mm diameter) containing 40 ml of the Modified Strullu-Romand (MSR) medium (Declerck *et al.*, 1998) lacking sucrose and vitamins and solidified with 3 g l^{-1} Phytagel™ (Sigma-Aldrich, Inc., St Louis, USA) and adjusted to pH 5.5 before sterilization (121°C for 15 min). Twenty-five seeds were placed on each Petri plate. The Petri plates were incubated in the dark at 27°C.

Seeds germinated within 1–2 days and plantlets were ready to use after 4 days.

4.3. Arbuscular mycorrhizal fungal culture

The AM fungus *Glomus* sp. MUCL 41833 was supplied by the Glomeromycota *in vitro* collection (GINCO – http://www.mycorrhiza.be/ginco-bel) on root organ cultures (ROC) of carrot (*Daucus carota* L.). The cultures were provided in Petri plates (90 mm diameter) on the MSR medium supplemented with 10 g l^{-1} sucrose, adjusted to pH 5.5 before sterilization (121°C for 15 min) and solidified with 3 g l^{-1} Phytagel™ (Cranenbrouck *et al.*, 2005). The Petri plates were incubated for 3 months in the dark in an inverted position at 27°C. Several thousand spores were produced within this period.

4.4. *Trichoderma harzianum* culture

A culture of *Trichoderma harzianum* Rifai MUCL 29707 was supplied by the Mycothèque de l'Université catholique de Louvain (MUCL) (http://bccm.belspo.be/about/mucl.php) on potato dextrose agar (PDA) medium (Scharlau Chemie S.A, Barcelona, Spain). A plug of gel containing several conidia and mycelium was placed into a sterile glass tube (1.5 ml) filled with 0.4 ml of 1% sterile distilled water-peptone (SDWP, Duchefa, The Netherlands). The plug was then swirled in the SDWP with a vortex mixer for 15 s and the suspension was serially diluted to 10^{-2}. From this dilution, 50 µl was spread on

fresh PDA medium (25 ml) in a Petri plate (90 mm diameter). The Petri plate was subsequently incubated 4 days in the dark at 25°C and afterwards at 4°C until needed.

4.5. Experimental set-up

A mycelium donor plant (MDP) *in vitro* culture system was set up for the fast and homogenous mycorrhization of potato plantlets following the methodology described by Voets *et al.* (2009). Briefly, bi-compartmented Petri plates (90 mm diameter) were used to physically separate a root compartment (RC) from a hyphal compartment (HC). Both compartments were filled with 25 ml of MSR medium without sucrose and vitamins and solidified with 3 g l^{-1} Phytagel™. Four-days old *M. truncatula* seedlings were transferred in the RC with their roots placed on the surface of the MSR medium and the shoots extending outside the Petri plates. The seedlings were inoculated with approximately 100 spores of *Glomus* sp. MUCL 41833, extracted from the AM fungal culture following solubilization of the MSR medium (Cranenbrouck *et al.*, 2005). The Petri plates were then sealed and incubated in a growth chamber set at 22/18°C (day/night) and illuminated for 16 h d^{-1} under a PPF of 300 µmol m^{-2} s^{-1}. The extraradical mycelium (ERM) developed profusely in the RC and crossed the plastic barrier to spread in the HC. After 8 weeks, two ten-days old potato plantlets were introduced in the HC of each MDP *in vitro* culture system with their roots in contact with the ERM network and their shoots extending outside the Petri plates. The Petri plates

were sealed and incubated under the same conditions as above. After twelve days of contact with the ERM network, the potato plantlets were removed from the MDP *in vitro* culture systems and transferred into quadri-compartmented culture plates 12.4 x 8.5 cm (each compartment 3.1 x 8.5 cm) (quadri-PERM, Greiner Bio-One, Kremsmünster, Austria) (Fig. 13). In these culture plates, only three compartments were used. The plastic barrier separating the first and the second compartment was removed with a scalpel. This enlarged compartment (6.2 x 8.5 cm) was filled with 50 ml of MSR medium lacking sucrose and vitamins, adjusted to pH 5.5 before sterilization (121°C for 15 min) and solidified with 3 g l^{-1} Phytagel™. In this compartment, one mycorrhized potato plantlet was placed with the roots on the MSR medium and the shoots extending outside the culture plate. This compartment was referred as the root compartment (RC). The other compartment was filled with 20 ml of MSR medium lacking sucrose and vitamins, adjusted to pH 5.5 before sterilization (121°C for 15 min) and solidified with 3 g l^{-1} Phytagel™. This compartment was referred as the hyphal compartment (HC) and was only accessible to the ERM of the AM fungus.

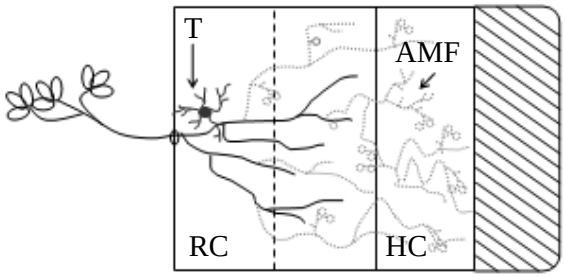

Figure 13 Schematic representation of the quadri-compartmented culture system (plate 12.4 x 8.5 cm) used to study the impact of *Trichoderma harzianum* MUCL 29707 on the transport of ^{33}P by the AM fungus *Glomus* sp. MUCL 41833. Only two compartments were used. An enlarged root compartment (RC; 6.2 x 8.5 cm) consisting of two merged compartments following the removal with a scalpel (dashed line) of the plastic barrier separating the first and the second compartment. Roots of an *in vitro* produced potato plantlet were associated to the AM fungus in this compartment, while the extraradical mycelium of the AM fungus was allowed to spread in a second compartment, named the hyphal compartment (HC; 3.1 x 8.5 cm). *T. harzianum* (T) was inoculated in the RC in close vicinity to the roots and in direct contact with the ERM of the AM fungus. The fourth compartment (dashed compartment) has been left empty.

The culture plates were wrapped with opaque plastic sheets and transferred to a growth chamber under the same controlled conditions as above. Potato roots crossing incidentally the plastic barrier were cut and removed using a scalpel and forceps. Five ml of MSR medium, lacking sucrose and vitamins, adjusted to pH 5.5 before sterilization (121°C for 15 min) and solidified with 3 g l^{-1} Phytagel™ was added weekly to the RC to provide the plants with nutrients and to maintain the medium at the level of the top of the partition wall, facilitating hyphae to cross from the RC to the HC. This medium was cooled to 40°C in a water bath before addition to avoid damages to the roots and AM fungus.

After a period of 4 weeks, the medium in the HC was covered by a profuse mycelium. This medium (containing the hyphae) was removed from the HC and replaced by 3 ml of fresh MSR medium, lacking sucrose and vitamins, adjusted to pH 5.5 before sterilization (121°C for 15 min) and solidified with 3 g l^{-1} Phytagel™. This favoured a homogenous re-growth of the ERM in the HC. After a period of 10 days, 44 culture systems were selected based of the number of spores and hyphal length produced in the RC and in the HC, estimated under a dissecting microscope (Olympus SZ40; Olympus Optical GmbH, Hamburg, Germany) at x 6.7-40 magnification following the method of Declerck *et al.* (1996a) and Giovannetti and Mosse (1980), respectively. In the HC, the surface covered by the ERM was also evaluated by tracing its surface on a transparent plastic sheet placed on the culture plate using a dissecting microscope. The surface area was subsequently cut out of the sheet and weighed. The surface of the HC covered by ERM was calculated by comparing to the weight of a transparent plastic sheet representing the full HC. The number of spores was 3171 ± 355 and the length of hyphae was 2587 ± 286 cm in the RC and 78 ± 49 spores and 1316 ± 309 cm covering 23 ± 4 cm^2 (91 ± 15%) of the HC. The number of leaves of the potato plantlets was also evaluated to 6 ± 1 leaves.

The 44 culture systems were divided in two homogenous groups of 22 systems based on the criteria above. One group (named the AMF treatment) contained potato plantlets associated to the AM fungus. The other group (named the AMF-T treatment) also contained

potato plantlets associated to the AM fungus but were additionally inoculated with *T. harzianum* (a 4 mm diameter plug of PDA medium containing several hyphae). The plug was carefully placed in the upper part of the RC in the vicinity of the roots and in direct contact with the ERM of the AM fungus (Fig. 13). The Petri plates were incubated horizontally in a growth chamber under the same conditions as above.

One experimental unit *i.e.* one replicate, consisted on an *in vitro* potato culture system inoculated with *Glomus* sp. MUCL 41833 alone or with the AM fungus and *T. harzianum* MUCL 29707.

4.6. Experiment 1: Impact of *T. harzianum* on the transport of ^{33}P by the AM fungus

Four days after the inoculation of *T. harzianum* in the RC, 12 Petri plates of each treatment (*i.e.* AMF and AMF-T treatments) were randomly selected among the two groups of 22 systems and a source of phosphorus (^{33}P) was added to the HCs. The source of ^{33}P was orthophosphate in diluted hydrochloric acid (<0.1M) supplied by PerkinElmer (Zaventem, Belgium). The radioactive solution was filter-sterilized (Acrodisc Syringe filters, PALL Corporation, Ann Arbor, MI, USA) and 100 µl was applied with a micropipette in each system on the whole surface of the medium in the HC allowing for a better distribution of ^{33}P via diffusion (Nielsen *et al.*, 2002). The activity of ^{33}P applied in each system was 49.99 KBq. A

formaldehyde-control was also considered. In 4 systems of each treatment, formaldehyde (2% v/v) was introduced in the HC two days before ^{33}P labelling in order to kill the AM fungus. This allowed determining whether the transport of ^{33}P was mediated by active fungal processes. Thus eight systems were considered for the AMF and AMF-T treatments without formaldehyde and 4 systems for the two treatments with formaldehyde. The 24 systems were incubated in a growth chamber under the same controlled conditions as above.

At harvest (*i.e.* 72 hours after labelling and 7 days after *T. harzianum* inoculation) the transport of ^{33}P was assessed in the fungal biomass of the RC (consisting of hyphae from both fungi in the AMF-T treatment and AM fungus alone in the AMF treatment) and HC (consisting of hyphae from the AM fungus in the AMF-T and AMF treatments), in the medium of the RC and HC, in the roots and in the shoots of *S. tuberosum*.

The shoots of the potato plantlets were harvested by cutting the stem at the level of the medium in the RC. The roots were removed from the medium and cleaned-free with milliQ water from the remaining gel and external hyphae. The medium of the RC and HC containing the fungal biomass were dissolved in citrate buffer (Cranenbrouck *et al.,* 2005) to separate the mycelium from the gel. The fungal biomass was then rinsed in sterile deionized water and collected. Aliquots of 5 ml of dissolved medium from the RC and HC were considered for ^{33}P analyses.

Shoot and root dry weights of plants were measured after three days at 70°C. The plant and fungal materials were then digested in 1 mL of HNO_3 (70%) and diluted to 10 ml with Milli Q water. An aliquot of 5 ml of the digested samples was then added to 15 ml of liquid scintillation cocktail (Ultima Gold^{TM} AB, Packard BioScience, Groningen, The Netherlands). Dissolved medium was also added to 15 ml of liquid scintillation cocktail. Samples were then counted for ^{33}P activity on a Packard Tri-Carb 1600CA liquid scintillation counter (Packard Instruments, Meriden, CT, USA).

4.7. Experiment 2: Impact of *T. harzianum* on hyphal metabolic activity and viability of the AM fungus

The 10 experimental units remaining from each treatment were randomly separated in two groups of 5 experimental units each. One group was harvested 4 days after *T. harzianum* inoculation and the other group 7 days after inoculation (corresponding to the timeline of ^{33}P labelling and harvest of Exp. 1, respectively). Before harvest, hyphal interactions between *Glomus* sp. and *T. harzianum* was assessed through direct observations under a bright-field light microscope (Olympus BH-2, Olympus Optical GmbH) at 50 X magnification, *i.e.* without disturbing the three dimensional configuration of the mycelium (De Jaeger *et al.*, 2010a). In addition, the number of spores, hyphal length produced in the RC and in the HC and the ERM covering the surface of the HC were estimated following the methods described above.

Plants were harvested and root colonization was assessed by staining according to the protocol described by Phillips and Hayman (1970). Colonization was estimated under a bright-field light microscope at x 50-250 magnification, following the method of McGonigle *et al.* (1990). For each subsample, at least 150 root intersections were assessed.

The mycelium of both fungi was carefully extracted following solubilization of the medium (Cranenbrouck *et al.*, 2005) of the RC and HC. The hyphae were stained for alkaline phosphatase (ALP) and succinate dehydrogenase (SDH) detection following the protocols of Olsson *et al.* (2002) and Saito (1995), respectively. For ALP activity, a 0.1 M Tris/HCl buffer (pH 8.5) with 1.1 mg ml^{-1} α-naphthyl phosphate Na salt (Sigma-Aldrich Inc. GmbH, Steinheim, Germany) and 1.0 mg ml^{-1} fast blue RR (Sigma-Aldrich Inc. GmbH, Steinheim, Germany) solution was used. Samples of mycelium were incubated for 90 min at room temperature in the staining solution and were washed with deionized water for 15 min. The hyphae were counterstained by immersion in 0.1% (w/v) acid fuchsin/lactic acid for 15 min and transferred to lactoglycerol for storage and de-staining. For SDH activity, samples of mycelium were incubated overnight at room temperature in a staining solution consisting of 0.05 M Tris/HCl buffer (pH 7.4), 0.5 mM $MgCl_2$, 0.25 M sodium succinate and 1 mg ml^{-1} Nitroblue tetrazolium (Sigma-Aldrich Inc. GmbH, Steinheim, Germany). Samples were washed with deionized water for 15 min. The hyphae were counterstained by immersion in 0.1% (w/v) acid

fuchsin/lactic acid for 15 min and transferred to lactoglycerol for storage and de-staining.

Mycelium samples stained for ALP or SDH activity were mounted on microscope slide with lactoglycerol. Granular precipitation of the samples was assessed with a bright-field light microscope (Olympus BH-2, Olympus Optical GmbH) at x 50 magnification. According to the magnified intersects method (McGonigle *et al.*, 1990), intersections of hyphae were classified as active or non-active according to visualization of granular precipitation. For each sample, at least 150 hyphal intersections were observed.

4.8. Statistical analysis

Data analysis was performed with the statistical software SAS Enterprise Guide 4. Data that were normally distributed and had homogeneous variances were subjected to an analysis of variance (ANOVA). The Tukey HSD (honest significant difference) test was used to identify the significant differences ($p \leq 0.05$).

5. Results

5.1. Experiment 1: Impact of *T. harzianum* on the transport of ^{33}P by the AM fungus

At the end of the experiment, 87.1 ± 2.7 and 92.5 ± 2.2% of the ^{33}P initially supplied in the HC was taken up by the AM fungus in the AMF and AMF-T treatments (*i.e.* 12.9 ± 2.7% and 7.5 ± 2.2%, remained in the medium), respectively. This difference was significant between treatments. The ^{33}P activity measured in the AM fungal biomass in the HC represented 58.5 ± 16.4 and 68.7 ± 15.4% of the ^{33}P taken up by the AM hyphae in the HC of the AMF and AMF-T treatments, respectively (Table 6). In the fungal biomass in the RC (consisting of AM mycelium in the AMF treatment and mycelium belonging to both fungi in the AMF-T treatment), the ^{33}P content represented 9.1 ± 2.2 and 9.4 ± 2.3% of ^{33}P taken up by the AM hyphae in the HC, respectively (Table 6). No significant difference in the ^{33}P activity or ^{33}P specific activity of the fungal biomass of the HC and the RC were observed between the treatments. Similarly, the ^{33}P content of the roots did not differ among the two treatments and represented 19.1 ± 7.6 and 15.1 ± 7.1% of ^{33}P taken up by the AM hyphae in the HC of the AMF and AMF-T treatments, respectively (Table 6). No significant difference in ^{33}P specific activity in the roots was observed between the treatments (Table 6).

Table 6 Phosphorus activities (cpm: count per minute) and specific activities measured on shoot and roots of *S. tuberosum*, fungal biomass (*i.e.* hyphae from AM fungus in the AMF treatment and from both fungi in the AMF-T treatment) in the root compartment (RC), medium in the root compartment, fungal biomass (*i.e.* hyphae from the AM fungus in the AMF-T and AMF treatments) in the hyphal compartment (HC) and medium in the hyphal compartment (HC).

Activity measured (cpm)	AMF		AMF-T	
Shoot	41779 ± 25512b	(8.5 ± 3.0)	13096 ± 3586a	(2.8 ± 1.8)
Roots	96221 ± 26685a	(19.1 ± 7.6)	71395 ± 28739a	(15.1 ± 7.1)
Fungal biomass (RC)	47312 ± 11883a	(9.1 ± 2.2)	45560 ± 11642a	(9.4 ± 2.3)
Fungal biomass (HC)	312455 ± 57497a	(58.5 ± 16.4)	314811 ± 57058a	(68.7 ± 15.4)
Medium (RC)	22921 ± 8224a	(4.5 ± 1,9)	18341 ± 7605a	(3.8 ± 1,4)
Medium (HC)	116102 ± 21202b		81176 ± 22873a	
Specific activity (cpm/mg)	AMF		AMF-T	
Shoot	264 ± 73a		76 ± 40b	
Roots	3447 ± 1538a		2223 ± 1396a	
Specific activity (cpm/cm)	AMF		AMF-T	
Fungal biomass (RC)	12 ± 3a		12 ± 3a	
Fungal biomass (HC)	195 ± 35a		218 ± 39a	

Activities in cpm ± standard deviations (n = 8). Values in parenthesis correspond to the percentages ± the standard deviations of the distribution of the ^{33}P total uptake by the AM fungus measured in the AM fungal biomass (*i.e.* AM mycelium in the hyphal compartment (HC) of the AMF and AMF-T treatments, AM mycelium in the root compartment (RC) of the AMF- treatment and in the fungal biomass of both fungi in the root compartment (RC) of the AMF-T treatment), and in the potato roots and shoots. Specific activity in cpm ± standard deviations (n = 8), estimated per mg of roots and shoots of potato plantlet or estimated per cm of hyphal length of AM fungal mycelium.For each activity, values in the same line followed by identical letters do not differ significantly (Tukey HSD test, $p \leq 0.05$).

To the contrary, the ^{33}P content measured in the shoots was significantly higher in the AMF treatment as compared to the AMF-T treatment and represented 8.5 ± 3.0 and 2.8 ± 1.8% of ^{33}P taken up by the AM hyphae in the HC, respectively (Table 6). A significant difference in ^{33}P specific activity in the shoot was identically observed between the treatments (Table 6). The ^{33}P released in the RC medium did not differ among the treatments and represented 4.5 ± 1.9 and 3.8 ± 1.5 of ^{33}P taken up by the AM hyphae in the HC for the AMF and AMF-T treatment, respectively (Table 6).

In the formaldehyde treatments, the ^{33}P remained in the HC and was neither detected in the fungal biomass or medium of the RC nor in the plant roots or shoots of the AMF and AMF-T treatments.

5.2. Experiment 2: Impact of *T. harzianum* on hyphal metabolic activity and viability of the AM fungus

In the AMF-T treatment, intermingled and overlapped hyphae of both fungi as well as mycoparasitism (*i.e.* coiling and penetration of the extraradical mycelium) of the AM fungal mycelium by *T. harzianum* were observed in the RC (data not show).

The AM development in the RC and in the HC did not significantly differ in presence or in absence of *T. harzianum*. Four days after *T. harzianum* inoculation (at ^{33}P labelling time), the AM mycelium covered the whole surface of the HC (*i.e.* 25.2 cm^2) in presence as well as in absence of the saprotrophic fungi. In the RC,

the number of spores was 4743 ± 453 and 4632 ± 735 and the length of hyphae was 3316 ± 374 cm and 3282 ± 567 in absence or in presence of *T. harzianum*, respectively. In the HC, the number of spores was 125 ± 35 and 90 ± 54 and the length of hyphae was 1485 ± 286 cm and 1407 ± 239 in absence or in presence of *T. harzianum*, respectively. At harvest (*i.e.* 7 days post *T. harzianum* inoculation and 3 days post ^{33}P labelling), in the RC, the number of spores was 5208 ± 351 and 5119 ± 583 and the length of hyphae was 3831 ± 314 cm and 3703 ± 545 cm in absence or in presence of *T. harzianum*, respectively. In the HC, the number of spores was 142 ± 69 and 93 ± 49 and the length of hyphae was 1598 ± 236 cm and 1439 ± 257 in absence or in presence of *T. harzianum*, respectively.

The presence of *T. harzianum* in the RC decreased significantly the mycorrhizal viability (SDH staining) in the RC and in the HC (Fig. 14a). The proportion of AM viable hyphae in the RC was reduced by 28.5% and 51.7% in the AMF-T treatment as compared to the AMF treatment 4 and 7 days after the inoculation of *T. harzianum*, respectively. In the HC, the proportion of AM viable hyphae was reduced by 8.9% and 14.4% in the AMF-T treatment as compared to the AMF treatment 4 and 7 days after *T. harzianum* inoculation respectively.

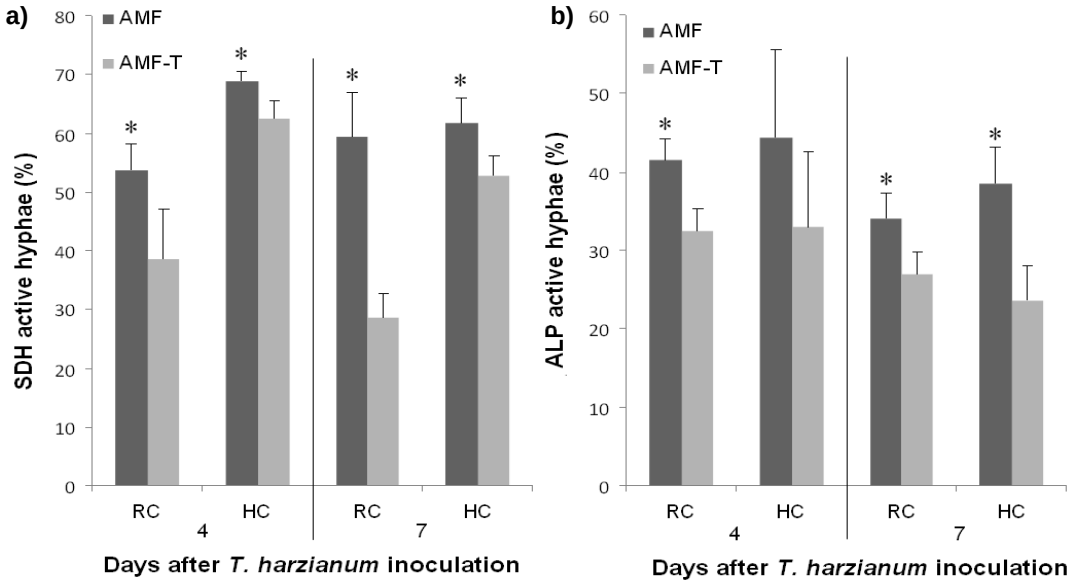

Figure 14 Proportion of metabolics activities determined in the extraradical hyphae of *Glomus* sp. MUCL 41833 in absence (AMF treatment) or in presence of *T. harzianum* MUCL 29707 (AMF-T treatment) (mean ± standard deviations, $n = 5$) 4 and 7 days after inoculation of the saprotrophic fungus. Analysis was conducted in the root compartment (RC – accessible to both fungi) and hyphal compartment (HC – only accessible to the AM fungus). **a)** Proportion of succinate dehydrogenase (SDH) activity. **b)** Proportion of alkaline phosphatase (ALP) activity. Asterisks indicate a significant difference ($p \leq 0.05$, Tukey HSD test) between the treatments.

Similarly, a significant difference was found in the mycorrhizal metabolic activity (ALP staining) between the two treatments (Fig. 14b). The presence of *T. harzianum* decreased the proportions of ALP-active hyphae in the RC but not in the HC, 4 days after *T. harzianum* inoculation. In the RC, the proportion of ALP-active hyphae of the AM fungus was reduced in the AMF-T treatment by 21.6 and 20.8% as compared to the AMF treatment 4 and 7 days after *T. harzianum* inoculation, respectively. In the HC, the proportion of ALP-active hyphae of the AM fungus was significantly different 7 days after *T. harzianum* inoculation and was reduced in the AMF-T treatment by 38.4% as compared to the AMF treatment.

Table 7 Root colonization of *S. tuberosum* (total, arbuscular and vesicular; McGonigle *et al.*, 1990) following inoculation by the AM fungus *Glomus* sp. MUCL 41833, 4 and 7 days after inoculation with *Trichoderma harzianum* MUCL 29707.

Time (days)	4		7	
	AMF	AMF-T	AMF	AMF-T
Total colonization (%)	56.5±8.2a	57.3±6.9a	62.6±12.4a	57.1±5.1a
Arbuscular colonization (%)	21.3±3.2a	24.0±4.1a	28.9±9.9a	25.4±2.3a
Vesicular colonization (%)	5.8±2.0a	6.6±3.8a	10.1±3.4a	9.0±1.5a

For each time (*i.e.* 4 or 7 days after *T. harzianum* inoculation), values in the same row followed by identical letters did not differ significantly at $p \leq 0.05$ (Tukey HSD test). Data represent the means of five replicates (mean ± standard deviations).

No difference in total potato root colonization, arbuscular and vesicular colonization were observed between the treatments during the experimental period (Table 7). The total root colonization 7 days after *T. harzianum* inoculation ranged from 62.6 ± 12.4% in the AMF treatment to 57.1 ± 5.1% in the AMF-T treatment. During the

experimental study, the arbuscular colonization increased from 21.3 ± 3.2% to 28.9 ± 9.% in absence of *T. harzianum* and from 24.0 ± 4.1 % to 25.4 ± 2.3% in presence of the saprotrophic fungi. The vesicular colonization raised from 5.8 ± 2.0% to 10.1 ± 3.4% in absence of *T. harzianum* and from 6.6 ± 3.6 to 9.0 ± 1.5% in its presence. Arbuscular and vesicular colonization were not significantly different between treatments.

6. Discussion

The interactions between mycorrhizal fungi and other soil organisms are complex. If beneficial interactions have been frequently mentioned (Bonfante and Anca, 2009), several authors have also reported antagonistic interactions with bacteria, fungi and microarthropods which may affect the functioning of the AM symbioses (Warnock *et al.*, 1982; Linderman, 1988; Paulitz and Linderman, 1991). Recently, De Jaeger *et al.* (2010a) demonstrated, under *in vitro* controlled conditions, that *T. harzianum* was able to impact the viability of AM fungi by feeding on its intra and extraradical mycelium. Therefore, it seemed conceivable that the disruption of this hyphal continuum from soil to plant could affect the bi-directional transport of C and P between both partners of the AM symbiosis. In our experimental conditions, ^{33}P was measured in hyphae, roots and shoots demonstrating the capacity of the AM fungus to take up, translocate and transfer P from a root-free compartment to its host plant in the absence as well as in the presence of *T. harzianum*. However, the amount of P transported was affected in presence of the saprotroph. More P was taken up by the AM fungus in presence of *T. harzianum*, while less P accumulated in the shoots of the potato plants. This observation was paralleled with a reduction of the viability (measured via SDH staining) and metabolic activity (measured via ALP staining) of the AM extraradical hyphae in presence of *T. harzianum*.

Radiotracer studies have demonstrated that phosphate uptake by the extraradical mycelium of the AM fungus is the very first step in the process of P transport to the plant (Jakobsen *et al.*, 1992). In the extraradical hyphae, P enters the cytoplasm via high-affinity $Pi:H^+$ symporters (Harrison and Van Buuren 1995; Maldonado-Mendoza *et al.* 2001; Javot *et al.* 2007) and is rapidly transferred to the fungal vacuole where it is polymerized to form poly-phosphate chains (poly-P) (Ezawa *et al.* 2001, 2004). The poly-P are then translocated along the hyphae to the intraradical mycelium and transferred to the plant cells (Harrison *et al.*, 2010). In our study, the proportion of ^{33}P remaining in the medium of the HC was significantly lower in presence of *T. harzianum* in the RC, a consequence of the higher uptake by the AM extraradical mycelium in presence of the saprotroph spatially separated from the ^{33}P source and AM mycelium in contact with ^{33}P. This enhanced uptake of P associated to an overall lower transport of P by the AM fungi to the host plant seems contradictory and exclude the uptake as being the cause for this lower transport of P. Purin and Rillig (2008) hypothesized that mycoparasitized AM fungi (*i.e.* by *T. harzianum*) may compensate metabolites loss or repair damaged tissue by increasing nutrient uptake from the environment (*i.e.* P, N uptake) or host plant (*i.e.* C acquisition), and thus could explain our observation. For instance, it could be suggested that in response to AM mycelium parasitism, the expression or activity of phosphate transporters could be increased. Regulation of P transporter gene expression coding for a protein belonging to the Pi/H^+ co-

transporters family in the extraradical hyphae appears to be a rapid and dynamic process, as indicated by the increase and decrease in P transporter gene transcript levels in response to a modification in the surrounding hyphal environment (Maldonado-Mendoza *et al.,* 2001; Olsson *et al.,* 2005) or in response to a signal from the host plant (Benedetto *et al.,* 2005). Maldonado-Mendoza *et al.* (2001) also demonstrated that a modification in the root compartment may regulate P uptake by the extraradical hyphae in the hyphal compartment, suggesting communication between the different parts of the mycelium subject to the differing environmental conditions (Hodge, 2010). Consequently, we hypothesized that *T. harzianum* may impact the uptake of P by the AM fungus even in its absence in the ^{33}P labelled compartment.

Due to parasitism of the extra and intraradical mycelium (De Jaeger *et al.*, 2010a), it could be expected that the impact of *T. harzianum* on P transport mainly concerned its translocation within the extra and intraradical hyphae. As demonstrated by the decrease of AM viability and metabolic activity in presence of the saprotroph, the parasitism of AM fungi resulted in a rapid degradation of the extraradical cytoplasmic content as earlier mentioned (Rousseau *et al.*, 1996; De Jaeger *et al.*, 2010a) indicating that *T. harzianum* was feeding on the AM fungus as reported by Benhamou and Chet (1997). This behaviour could have led to the acquisition by *T. harzianum* of P present in the cytoplasm of the AM fungus leading to a decrease in P reaching the host plant. Although the ^{33}P activity in the biomass of the

two fungi could not be separated, it seems reasonable to assume that *T. harzianum* could have derived part of the P present in the mycelium of the AM fungus.

The alteration of the integrity of the mycorrhizal network could have another direct effect on the translocation of P and subsequently on its transfer to plants. Indeed, by isolating hyphal sections and interrupting cytoplasm continuity within and between the extra and intraradical hyphae, the capacity of the AM fungus to translocate P would be reduced. In a previous study, De Jaeger *et al.* (2010a) observed that *T. harzianum* preferentially penetrated and colonized runner hyphae (~65% of damaged hyphae), while lower order branching hyphae were less concerned. As runner hyphae represent conduits mobilizing the resources (*i.e.* P, and C) from and to lower order hyphae (Smith and Read, 2008), their parasitism may partly block or slow down the translocation of P towards and within the intraradical hyphae of the AM fungus. Similar results were obtained during interactions between fungal grazers and AM fungi. By grazing on the extraradical mycelium of AM fungi, mycophage animals such as Collembola affected the functionality of AM symbiosis (Hattingh *et al.*, 1973, Warnock *et al.*, 1982; Fitter and Sanders, 1992; Fitter and Garbaye, 1994). For instance, Warnock *et al.* (1982) reported a reduction in P inflow to roots from 6.8 10^{-14} to 1.7 10^{-14} mol cm^{-1} s^{-1} due to the grazing of Collembola on the extraradical mycelium of an AM fungus. Interestingly, Johnson *et al.*, (2005) showed that Collembola could reduce by 32% the

mycorrhizosphere respiration indicating that grazing also disrupted the translocation of C towards the extraradical phase of the AM fungi. Although no measurement was made on C flow in presence of *T. harzianum* in our experiment, we may suspect a similar decrease related to the disruption of the extra and intraradical hyphae reported above. It is to be noted that a lower translocation of C to the AM extraradical mycelium could partly explain the decrease in AM viability (SDH staining) and activity (ALP staining) noted in presence of *T. harzianum* in the RC and HC.

It is well known that arbuscules are the major site for the transfer of minerals and carbohydrates between both partners of the symbiosis (Smith and Read, 2008). Despite the impact of *T. harzianum* on *Glomus* sp., no differences were observed in the proportion of arbuscules, or of other intraradical structures (*i.e.* vesicles) in the potato roots. This is possibly due because the AM symbiosis was established prior to the introduction of the saprotrophic fungus as earlier reported (McAllister *et al.*, 1994b; Vasquez *et al.*, 2000). In similar experimental conditions, De Jaeger *et al.* (2010a) demonstrated that *T. harzianum* was able to affect the intraradical hyphae viability of *Glomus* sp. without decreasing the AM fungal development inside the roots. Smith and Dickson (1991) have shown that the number of SDH active arbuscules could reflect a surface area for nutrient exchange between living symbionts and therefore symbiotic efficiency. Positive correlation between arbuscules ALP activity and the mycorrhizal responses was also obtained by Guillemin

et al. (1995). Thus, a decrease of active arbuscules due to the presence of *Trichoderma* sp. may affect the nutrient transfer from the fungal cells to the host cells, and interestingly the C acquisition by the AM fungus, hypothesis that should be considered in future experiments.

Taken together, the results obtained in this study suggested that *T. harzianum* could impact the functioning of the symbiosis by decreasing the capacity of the AM fungus to translocate and transfer P to its host plant. Given the importance of mycorrhizal mycelia networks for the establishment, diversity, nutrition and productivity of plant communities (van der Heijden *et al.* 2008) their disruption by soil microorganisms may have a wide range of additional effects that need to be investigated. In the case that AM extraradical hyphae are parasitized near to the root surface interrupting the hyphal continuity with the intraradical mycelium, relatively low levels of hyphal predation could have dramatic effects on mycorrhizal function. As suggested by Fitter and Sanders (1992) and Gange (2000), the AM intraradical structure would still receive carbon compounds from the plant host but the reciprocal transaction, *i.e.* the transport of nutrient to the roots would be prevented. In this situation, the relationship between the AM fungi and their host plant would become parasitic rather than mutualistic (Johnson *et al.*, 1997), a condition that causes reduction of plant growth and yield (Klironomos, 2003; Lendzemo *et al.*, 2004).

In this study, we reported for the first time the impact of *T. harzianum* on the transport of P by an AM fungus. An increased uptake was observed, as a possible stress reaction caused by mycoparasitism of AM mycelium, while the transfer of P to the host plant was significantly reduced. This reduction in plant P acquisition could be explained by the disruption of the external and internal hyphae continuity and decrease in SDH and ALP activities, affecting the P translocation within the AM fungal mycelium. The antagonistic effects of *T. harzianum* on *Glomus* sp. may thus have important effects on the growth and function of the AM fungi and also affect the plant performance indirectly by decreasing the source-sink relationship between the AM fungi and its host plant.

Acknowledgements This research was sponsored by the Direction générale opérationnelle de l'Agriculture, des Ressources naturelles et de l'Environnement du service public de Wallonie under contract number D31-1193, entitled 'Valorisation de la microflore bénéfique des sols pour le contrôle de la flore pathogène des productions de pomme de terre comme alternative à l'utilisation des pesticides'. HB acknowledges the FRS-FNRS grant as a Research Fellow (Chargé de Recherche). The authors thank Dr I. van Aarle for technical advice and helpful comments. Thanks are also addressed to Prof. E. Smolders and his team (Division of Soil and Water Management, KULeuven, Belgium) for ^{33}P activity measurements.

VIII. CHAPTER 5

Co-entrapment of *Trichoderma harzianum* and *Glomus* sp. within alginate beads: impact on the arbuscular mycorrhizal fungi life cycle

Submitted to Journal of Applied Microbiology

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1. Preface

In **Chapter 3** and **4** we focused on the effect of *T. harzianum* on the symbiotic phase of the AM fungi. We observed that *T. harzianum* affected the viability and the functionality of the AM symbiosis when the saprotrophic fungus was inoculated on AM established networks. However, several studies demonstrated that the effects of saprotrophic fungi on AM symbiosis may differ widely depending on the inoculation timing of both microorganisms (McAllister *et al.*, 1994b). Based on this assumption we evaluated in **Chapter 5** whether *T. harzianum* affected the AM establishment and fungal development when both microorganisms were inoculated at the same time. The entrapment in alginate beads was chosen as a suitable cell-carrier as earlier reported for several microorganisms including *T. harzianum* (Fravel *et al.*, 1985) and AM fungi (Declerck *et al.*, 1996b; Plenchette and Strullu, 2003).

2. Summary

Aims: This study was performed to explore the compatibility and applicability of beneficial microorganisms (i.e. *Trichoderma harzianum* MUCL 29707 and *Glomus sp.* MUCL 41833) co-entrapped in alginate beads.

Methods and Results: Spores of *Glomus sp.* and conidia of *T. harzianum* were immobilized in alginate beads and the impacts of the saprotrophic fungi on the pre-symbiotic and symbiotic phase of the arbuscular mycorrhizal (AM) fungi evaluated under strict *in vitro* culture conditions. Our results demonstrated the capacity of both microorganisms in combination to re-growth outside the calcium-alginate coating. The presence of *T. harzianum* did not hinder the AM fungal development but rather stimulate its spore production and fitness.

Conclusions: The combination of *T. harzianum* (MUCL 29707) with *Glomus* sp. MUCL 41833, in alginate beads may represent a reliable alternative inoculum formulation for application in sustainable agriculture.

Significance and Impact of the Study: The entrapment in alginate beads of two fungi (i.e. a saprotroph and a symbiont) having beneficial effects on plants, represents a promising formulation for the development of inoculants adapted to field application.

Key words: alginate-entrapment, arbuscular mycorrhizal fungi, *in vitro* culture, Malthusian fitness, succinate dehydrogenase, *Trichoderma harzianum*.

3. Introduction

Microbes represent the unseen majority in soil and influence directly and indirectly the biodiversity, productivity, composition of plant communities and ecosystem functioning (van der Heijden *et al.*, 2008). In the last decades, soil microorganisms have been described, characterized, and tested for their use as plant growth promoters and biocontrol agents (Kaewchai *et al.*, 2009). Among these microorganisms, an abundant literature has reported the beneficial effects of arbuscular mycorrhizal (AM) fungi (van der Heijden *et al.*, 2008) and of saprotrophic fungi from the genus *Trichoderma* (Harman *et al.*, 2004), on plant growth and protection against biotic stresses. AM fungi which colonizes the roots of nearly 80% of terrestrial plants (Smith and Read, 2008) have been shown to increase plant performance and yield (van der Heijden, 2008), to improve soil quality (Rillig and Mummey, 2006) and to reduce the damages caused by soil-borne pathogens (Whipps, 2004). Identically, *Trichoderma* spp. have been reported to control a broad spectrum of fungal pathogens via the combination of several mechanisms among which mycoparasitism and antibiosis (Harman *et al.*, 2004). Both microorganisms in combination may thus represent a reliable alternative to reduce the application of pesticides in agro-environments. However, their broad-scale field application necessitates the development of adequate carriers allowing their advantageous combination (Bashan, 1998).

Among the formulations frequently reported, the immobilization of cells within beads has been frequently mentioned for agricultural and environmental applications (see review by Bashan, 1998). Immobilization (encapsulation/entrapment) within beads offers an excellent protection of cells against many biotic and abiotic environmental constraints (Bashan, 1998) and increases the microbial survival and thus shelf life following inoculation into the field (Vassilev *et al.*, 2001).

One of the most common materials used for the immobilization of microorganisms is alginate, a natural, biodegradable polymer (Bashan, 1998). Alginate-entrapment methods have been used for the immobilization of AM fungi (Declerck *et al.*, 1996b) and *Trichoderma* sp. (Fravel *et al.*, 1985) alone but no studies reported yet the co-entrapment of both microorganisms together.

In the last years, several studies have mentioned the beneficial effects of mixed non-entrapped inocula of both microorganisms on plant growth (Calvet *et al.*, 1993) as well as on the control of various plant diseases (Chandanie *et al.*, 2009). Interestingly, De Jaeger *et al.* (2010a) also reported mycoparasitism of *Trichoderma* spp. over AM fungi. As a consequence, the compatibility of AM fungi with *Trichoderma* spp. within beads should first be evaluated to guarantee the successful application of the co-entrapment technology.

Within this context, *in vitro* culture systems provide useful and powerful experimental tools to allow direct and non-perturbed microscopic observations of the interactions between microorganisms (De Jaeger *et al.*, 2010a). Using *in vitro* culture systems, the interaction between *Glomus* sp. MUCL 41833 and *T. harzianum* MUCL 29707 co-entrapped in alginate beads was investigated to determine whether (i) the co-entrapment process impacted the pre-symbiotic phase of AM fungi and (ii) *T. harzianum* influences the symbiotic AM fungal development.

4. Materials and methods

4.1. Potato plantlets

In vitro propagated potato plantlets (*Solanum tuberosum* L., var. Bintje) were supplied by the Station de Haute Belgique in Libramont, Belgium. Plantlets were micro-propagated every 5-6 weeks by placing nodal cuttings in sterile culture boxes (90 × 60 × 50 mm, 20 cuttings per box) containing 50 ml of Murashige and Skoog (MS) medium following the method described by Voets *et al.* (2005). The plantlets were kept in a growth chamber set at 21 °C with a photoperiod of 16 h day^{-1} and a photosynthetic photon flux (PPF) of 300 µmol m^{-2} s^{-1}.

4.2. Arbuscular mycorrhizal fungi

The AM fungus *Glomus* sp. MUCL 41833 was supplied by the Glomeromycota *in vitro* collection (GINCO – http://www.mbla.ucl.ac.be/ginco-bel) on root organ cultures (ROC) of carrot (*Daucus carota* L.). The cultures were provided in Petri plates (90 mm diameter) on the Modified Strullu-Romand (MSR) medium supplemented with 10 g l^{-1} sucrose, adjusted to pH 5.5 before sterilization (121 °C for 15 min) and solidified with 3 g l^{-1} Phytagel™ (Sigma-Aldrich, Inc., St Louis, USA) (Cranenbrouck *et al.* 2005). The Petri plates were incubated during 3 months in the dark in an inverted position at 27 °C.

4.3. *Trichoderma harzianum*

A culture of *Trichoderma harzianum* Rifai MUCL 29707 was supplied by the Mycothèque de l'Université catholique de Louvain (MUCL – http://bccm.belspo.be/about/mucl.php) on potato dextrose agar (PDA) (Scharlau Chemie S.A, Barcelona, Spain). A plug of gel containing several conidia and mycelium was placed into a sterile glass tube (1.5 ml) filled with 0.4 ml of 1% sterile distilled water-peptone (SDWP) (Duchefa, The Netherlands). The plug was swirled in the SDWP with a vortex mixer for 15 seconds and 50 µl of the suspension was spread on PDA. The Petri plates were incubated four days in the dark at 25°C and subsequently maintained at 4°C until needed.

4.4. Entrapment process of the AM fungus and *T. harzianum* in alginate beads

A solution of sodium alginate 20 g l^{-1} (Sigma-Aldrich, Inc., St Louis, USA) was prepared by dissolution at 60°C before autoclaving for 15 min at 121°C.

Spores of the AM fungus were isolated from the ROC by solubilization of the MSR medium (Donner and Bécard, 1991). The spores were subsequently maintained in deionized sterilized water (121°C for 15 min). Cluster of spores were separated with sterile needles under a dissecting microscope (Olympus SZ40, Olympus Optical GmbH, Hamburg, Germany) at 6.7-40x magnification. The

isolated spores were then re-suspended in the sodium alginate solution. The number of spores in the suspension was quantified under a bright-field light microscope (Olympus BH-2, Olympus Optical GmbH, Hamburg, Germany) at 50-250x magnification and their concentration was adjusted to 300 individuals per ml of sodium alginate solution.

Conidia of *T. harzianum* were extracted from the PDA medium by adding 10 ml of deionized sterilized water on the surface of the fungal culture. The Petri plates were gently shacked for 5 min and a suspension was sampled with a micropipette. Conidial suspension was quantified in a Thoma chamber (Fisher Scientific, Pittsburg, USA) under a bright-field light microscope at 50-250x magnification. A fraction of the conidial suspension was added to the sodium alginate solution under sterile conditions to obtain a final concentration of 150 conidia per ml of the sodium alginate solution.

For the co-entrapment process, spores of the AM fungus and conidia of *T. harzianum* were added to the same solution of sodium alginate following the method described above to have in final concentration 300 spores of the AM fungus and 150 conidia of *T. harzianum* per ml of sodium alginate solution.

The sodium alginate solutions (10 ml) containing spores of the AM fungus, conidia of *T. harzianum* or a combination of both microorganisms were maintained under agitation in flasks. Droplets of

33 µl were sampled with a micropipette (Gilson, Middleton, USA) and subsequently added into a 0.1 M solution of sterilized (121 °C for 15 min) $CaCl_2$ under constant agitation for polymerisation. The beads were maintained under agitation for 30 minutes (Declerck *et al.*, 1996b). The alginate beads were thereafter collected on a 100 µm sterilized nylon mesh, washed with deionized sterile water and maintained at 4° C in a Petri plate. Each alginate bead contained an approximate of 10 spores of the AM fungus (AMF-treatment), 5 conidia of *T. harzianum* (T-treatment) or a mixture of 10 spores and 5 conidia of both fungi (AMF-T treatment).

4.5. Expriment 1: Evaluation of the percentage of potentially infective beads (%PIB) following co-entrapment of both microorganisms

MSR medium lacking sucrose and vitamins (Voets *et al.*, 2005) was sterilized at 121°C for 15 min and subsequently cooled in a water bath to approximately 35°C. The MSR medium was poured in the Petri plate (50 ml/plate) and five beads belonging either to the AMF-treatment, T-treatment or AMF-T treatment were embedded into the MSR medium before solidification. Petri plates were then incubated at 27 °C in the dark in a completely randomized block design. Fifty replicates (*i.e.* beads) were considered per treatment.

Beads containing either the two fungi (AMF-T treatment) or one fungus (AMF-treatment and T-treatment) were examined under a

bright-field light microscope at 50-250x magnifications in order to assess the percentage of potentially infective beads (%PIB). The %PIB was originally developed by Declerck *et al.* (1996b) to evaluate the percentage of beads containing at least one germinated spore of AM fungi. This parameter was adapted in our study by evaluating the percentage of beads in which, at least, one germinated spore (%PIB-AMF) or conidia (%PIB-T) crossed the calcium-alginate coating. In the case of bead containing both fungi, the %PIB was calculated as above for each organism individually (%PIB-AMF' and %PIB-T') as well as for both organisms in combination (%PIB-AMF-T, in this case the calcium alginate coating should be crossed by both organisms). The %PIB AMF-T, %PIB-AMF, %PIB-AMF', %PIB-T and %PIB-T' were plotted against time at 2, 3, 6 and 14 days.

4.6. Experiment 2: Impact of *T. harzianum* on the AM fungus life cycle.

Similarly to experiment 1, five beads belonging either to the AMF-treatment, T-treatment or AMF-T treatment were embedded into the MSR medium on a Petri plate. A ten-day-old *in vitro* produced potato plantlet was subsequently inserted in the Petri plate following the protocol described by Voets *et al.* (2005). Petri plates were covered with an opaque plastic bag and incubated horizontally in a growth chamber set at 20/16 °C (day/night) with 70% relative humidity, a 16 h photoperiod and a PPF of 300 $\mu mol\ m^{-2}\ s^{-1}$ in a completely randomized block design. Ten ml of MSR medium lacking sucrose

and vitamins were added every three weeks in the Petri plates to maintain an adequate level of the MSR medium and to supply the plantlets with nutrients. Thirty five replicates (*i.e.* Petri plates containing each 5 beads) were considered per treatment (*i.e.* AMF, AMF-T and T).

4.6.1. Evaluation of the %PIB in presence of potato plantlet

Beads containing either the two fungi (AMF-T treatment) or one fungus (AMF-treatment and T-treatment) were associated with potato plantlets and examined for the percentage of potentially infective beads (%PIB-T, %PIB-T', %PIB-AMF, %PIB-AMF', %PIB-AMF-T) at 14 days, following the method described in experiment 1. Ten replicates per treatment were selected randomly among the 35 above. One experimental unit, *i.e.* one replicate, consisted of a ten-day-old potato plantlet inoculated with 5 beads containing both fungi (AMF-T treatment) or a single fungus (AMF-treatment or T-treatment).

4.6.2. Malthusian fitness

In order to measure the survival success and reproductive effort of the AM fungus in presence of *T. harzianum*, the Malthusian fitness (MF) was calculated (Pringle and Taylor, 2002). The MF is a measure of the instantaneous change in individual numbers over time in relation to the starting number of individuals.

It is measured by comparing the number of spores at an initial time (N_0), to the number of spores at a future time (N_t) as follows:

$$MF = [\ln(N_t/N_0)]/t$$

The N_0 is the number of spores contained in each Petri plate at the inoculation time (*e.g.* 5 alginate beads containing each 10 spores = 50 spores) and the Nt corresponded to the number of spores measured at 4, 8, 12, 16 and 20 weeks after alginate beads association to a potato plantlet.

The number of newly-produced spores of the AM fungus was estimated under a dissecting microscope at 6.7-40x magnifications following the protocol described in Voets *et al.* (2005). A grid of lines was marked on the bottom of each Petri plate. Spores were counted in each cell formed by the grid and summed over the entire Petri plate. The evaluation of the MF was conducted on the same Petri plates than those used in section 4.6.1.

4.6.3. Root colonization and mycelium viability assessment

In the AMF-T and AMF treatments, the twenty-five remaining Petri plates were randomly divided into 5 groups of five replicates. Roots and extraradical mycelium were harvested at 4, 8, 12, 16 and 20 weeks after alginate beads inoculation.

Root colonization was assessed by staining according to the protocol described by Phillips and Hayman (1970) and colonization was estimated under the bright-field light microscope, following the method of McGonigle *et al*. (1990).

For the assessment of the extraradical mycelium viability, hyphae of both fungi were carefully extracted following solubilisation of the MSR medium (see above). The ERM viability was estimated using the succinate dehydrogenase (SDH) staining technique (Saito, 1995). Samples were incubated overnight at room temperature in the staining solution, washed with deionised water during 15 minutes and immersed in 0.1% (w/v) acid fuchsin/lactic acid (Olsson *et al.*, 2002) during 15 min. Hyphae were mounted on slides in 100% glycerol. The SDH activity was counted under a bright-field light microscope using the magnified intersect method (McGonicle *et al.*, 1990). The hyphae were classified in SDH active and SDH inactive hyphae, corresponding to dark granular staining or without staining, respectively. This measure has been used to evaluate the antagonistic effect of *T. harzianum* on the AM fungi (De Jaeger *et al.*, 2010a).

4.6.4. Spore germination and continuous culture.

After 20 weeks, spores of the AM fungus isolated from five Petri plates of the AMF-T and AMF-treatments used in sections 4.6.1 were tested for their capacity to germinate and their ability to reproduce the

fungal life cycle following their association to potato plantlets under *in vitr*o culture conditions.

Fifty spores were extracted from the AMF-T and AMF- treatments following solubilisation of the MSR medium and placed in Petri plates (*i.e.* ten isolated spores per Petri plate) containing the MSR medium lacking sucrose and vitamins and subsequently associated to a ten-day old potato plantlet following the protocol described by De Jaeger *et al.* (2010b).

In the AMF-T treatment, AM spores and conidia of *T. harzanium* were both present in the solubilised medium. As it was not possible to separate both fungi and isolate the AM fungal spores from the conidia of *T. harzianum*, a third treatment (AMF (+T) treatment) was included to discriminate the effect of the origin of the spore from the influence of *T. harzianum*. This treatment associated extracted spores sampled from the AMF treatment and conidial suspension of *T. harzianum*. During the MSR medium solubilisation of AMF treatment a fraction of conidial suspension extracted from the fungal culture on PDA medium as described in section 2.4 was added to obtain the same concentration than measured with Thoma in the solubilisation solution of the AMF-T treatment. Fifty spores were associated with potato plantlet as described previously.

Petri plates were incubated under the same conditions as described above. Spores were examined under a bright-field light

microscope at 50-250x magnifications for germination at days 3, 7 and 11. Germination was considered when a hyphal tube emerging through the lumen of the subtending hyphae was observed.

For each treatment, the life cycle of the AM fungus was followed during 6 weeks. At that time, root colonization was assessed according to the method described above.

One experimental unit, *i.e.* one replicate, consisted of a ten-day-old potato plantlet associated with each treatment (*i.e.* AMF, AMF-T, AMF (+T) treatments). Five experimental units were considered for each treatment.

4.7. Microscopic analysis

Images of the pre-symbiotic and symbiotic phases of the AM fungus were captured with a digital camera (Leica DFC320; Leica Microsystems ltd) coupled to a compound bright-field light microscope and displayed on a computer using an image manager software (Leica IM50, version 4.0 Leica Microsystems Imaging solutions Ltd, Cambridge, United Kingdom).

4.8. Statistical analysis

Data analysis was performed with the statistical software SAS Enterprise Guide 4. Data that were normally distributed and had homogeneous variances were subjected to an analysis of variance (ANOVA). The Tukey HSD (honest significant difference) test was used to identify the significant differences ($p \leq 0.05$).

5. Results

5.1. Experiment 1: Evaluation of the percentage of potentially infective beads following co-entrapment of both microorganisms

Spores of *Glomus* sp. MUCL 41833 and conidia of *T. harzianum* MUCL 29707 entrapped in alginate beads were able to germinate and to re-grow outside the calcium-alginate coating either when grown alone or in combination (Table 8).

Table 8. Percentage of potentially infective beads (%PIB), *i.e.* containing at least one germinated spore (%PIB-AMF) or conidia (%PIB-T) crossing the calcium-alginate coating. In the case of bead containing both fungi, the %PIB was calculated as above for each organism individually (%PIB-AMF' and %PIB-T') as well as for both organisms in combination (%PIB-AMF-T). The % PIB AMF-T, %PIB-AMF, %PIB-AMF', %PIB-T and %PIB-T' were plotted against time at 2, 3, 6 and 14 days.

Time (days)	2	3	6	14
%PIB-T	72.0±4.5a	86.0±3.5a	96.0±1.9a	96.0±1.9a
%PIB-AMF	0.0±0.0b	10.0±3.0b	68.0±4.7b	100.0±0.0a
%PIB-T'	80.0±4.0a	82.0±2.5a	94.0±2.4a	94.0±2.4a
%PIB-AMF'	0.0±0.0b	14.0±3.5b	84.0± 3.7c	98.0±1.4a
%PIB-AMF-T	0.0±0.0b	12.0±3.5b	86.0±3.7c	96.0±1.4a

Data represent means of ten replicates, *i.e.* a Petri plate containing five beads (mean ± standard deviations). For each time of observation (*i.e.* 2, 3, 6, 14 days following entrapment), values in the same column followed by identical letter did not differ significantly ($p \leq 0.05$, Tukey test).

The first beads showing hyphal regrowth outside the alginate coat were observed at day 3 with the AM fungus (Fig. 15a) (in presence as well as in absence of the saprotroph) and at day 2 with *T. harzianum* (Fig. 15b) (in presence as well as in absence of the AM fungus). In the

co-entrapment treatment, overlapping hyphae of *T. harzianum* and *Glomus* sp. were observed, but mycoparasitism by the saprotrophic fungus on the AM fungus was never noted nor on the spores neither on the hyphae.

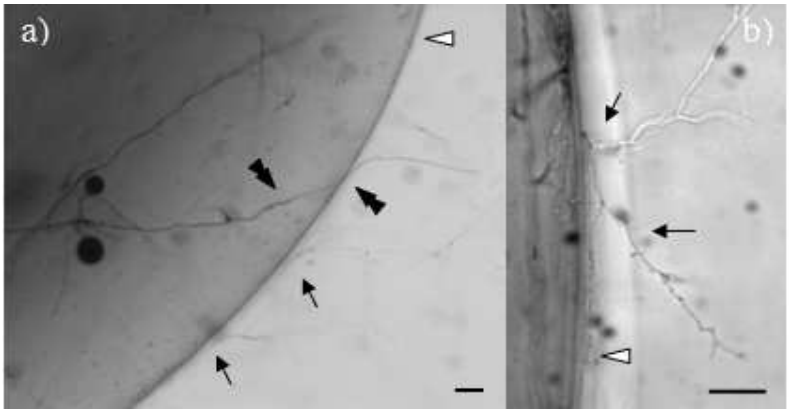

Figure 15 a) Regrowth of mycelium of *T. harzianum* (arrow) and germination of spore of the AM fungus (double arrowhead) extending out of the alginate polymer coating of the bead (empty arrowhead). Scale bar = 70 µm. **b)** Focus of regrowth of mycelium of *T. harzianum* (arrow) through the alginate polymer coating of the bead (empty arrowhead). Scale bar = 70 µm.

The %PIB estimated on the entrapped conidia of *T. harzianum* was already high at day 2, reached a maximum at day 6 and remained unchanged thereafter (i.e. %PIB-T and %PIB-T' – Table 8). In contrast, the %PIB estimated on the entrapped spores of *Glomus* sp. remained low until day 3, increased markedly at day 6 and reached a maximum at day 14 (i.e. %PIB-AMF and %PIB-AMF' – Table 8). A similar trend was observed for the %PIB estimated on both fungi in the co-entrapment treatment (%PIB-AMF-T –Table 8). Considering each fungus individually, no significant differences in the hyphal re-growth of *T. harzianum* was noted in absence (%PIB-T) or in presence

of the AM fungus (%PIB-T') during the 14 days of observation. Similarly, the re-growth of hyphae of the AM fungus did not differ in absence (%PIB-AMF) or in presence (%PIB-AMF-T) of *T. harzianum*, with the exception of day 6, where the hyphal re-growth of the AM fungus was significantly higher in the co-entrapped treatment (%PIB-AMF') as compared to the treatment where the AM fungus was entrapped alone (%PIB-AMF). Considering concomitant re-growth of both microorganisms (%PIB-AMF-T) we noted a significant difference with the %PIB-T at day 2, 3 and 6. At day 6, the %PIB-AMF was significant lower as compared to the %PIB-AMF-T. At day 14, no significant differences among the treatments (%PIB-AMF, %PIB-AMF-T and %PIB-T) were observed (Table 8).

5.2. Experiment 2: Impact of *T. harzianum* on the AM fungus life cycle

Following association with potato plantlets, the conidia of *T. harzianum* entrapped in beads (i.e. AMF-T and T treatments) started to germinate and re-grow outside the calcium-alginate coating at day one, while the spores of the AM fungus (i.e. AMF and AMF-T treatments) started to germinate within 2 days and crossed the alginate coating after 3 days. No significant differences in %PIB were observed at day 14 between the treatments. The values ranged from 98.0 ± 2.4% in the AMF-T treatment to 100.0% in the AMF treatment. The %PIB-T also reached 96.0 ± 1.9% and did not differ from the %PIB in the AMF-T treatment (98.0 ± 2.4%). The %PIB-T' and

%PIB-AMF' in the co-entrapment treatment reached 98.0 ± 2.4% and 98.0 ± 1.6%, respectively.

In the AMF and AMF-T treatments, the first contacts between the AM fungal hyphae and the potato roots were observed within 6 to 14 days in absence as well as in presence of *T. harzianum*. After 4 weeks of culture, some AM hyphae developed in the MSR medium. At week 8, a network of AM hyphae covered the whole surface of the MSR medium. Runner hyphae (RH) ramifying into secondary and lower order hyphae bearing hundreds of spores and branched absorbing structures (BAS) were observed.

In the T and AMF-T treatments, *T. harzianum* developed a network of highly branched and septated hyaline hyphae bearing hundreds of conidiophores covering the whole surface of the MSR medium within 7 days. The presence of the AM fungus did not impact the development of the saprotroph.

In the AMF-T treatment, intermingled and overlapped mycelia of both fungi as well as mycoparasitism (i.e. coiling and penetration of the extraradical mycelium) of the AM fungal mycelium were observed. *T. harzianum* was detected in the intraradical mycelium of the AM fungus (i.e. hyphae and vesicles) and formed peloton of saprotrophic hyphae in the roots (Data not shown).

The percentage of SDH activity measured in the hyphae of the AM fungus decreased with time but did not differ significantly in

presence or in absence of *T. harzianum* (Fig. 16). In week 20, values ranged from 55.7 ± 4.6% in the AMF-T treatment to 58.7 ± 5.0% in the AMF treatment.

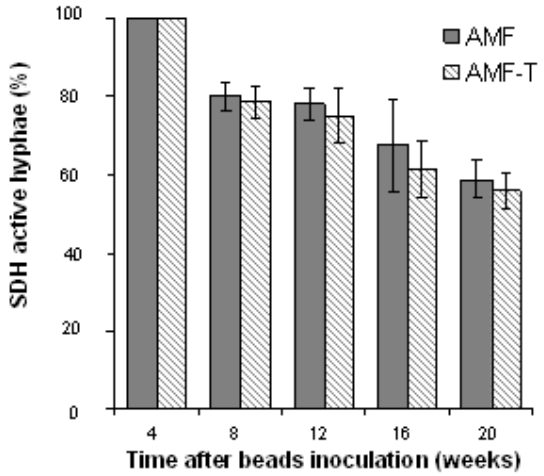

Figure 16. Influence of *T. harzianum* on the viability of the AM fungus measured by the percentage of succinate dehydrogenase (SDH) active extraradical hyphae of the AM fungi. AMF represents the AM fungus alone, AMF-T represents the AM fungus associated with *T. harzianum*. Data represent means of five replicates. The bars indicate standard deviations.

Whatever the treatment (AMF and AMF-T), the AM fungal spore production increased over time and the sporulation dynamics followed a sigmoidal curve (Fig. 17). No significant difference in the spore production between the treatments was observed during the first eight weeks. Thereafter, a marked stimulation of AM fungal spore production was observed in presence of *T. harzianum*. Values ranged from 2767 ± 938 spores in the AMF treatment to 11702 ± 3086 spores in the AMF-T treatment at week 12 and from 6867 ± 1594 spores in

the AMF treatment to 24461 ± 3306 spores in the AMF-T treatment at week 20.

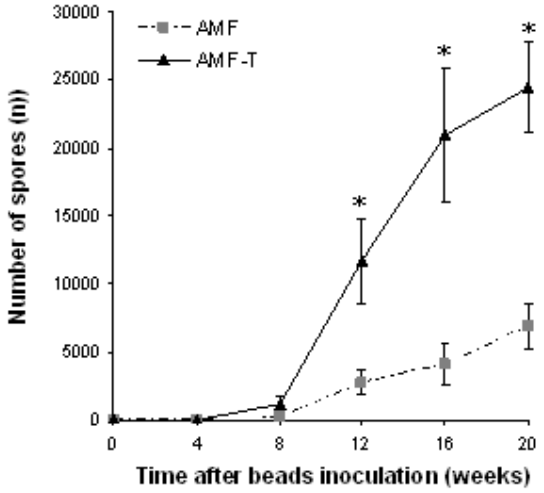

Figure 17 Dynamics of spore production of the AM fungus associated to autotrophic an potato plantlet (*S. tuberosum* cv. Bintje), in presence (AM-T treatment) or in absence of (AM-treatment) of *T. harzianum*. Data represent means of ten replicates (mean ± standard deviations). Column followed by * differed significantly between treatments at $p \leq 0.05$ (Tukey test).

The total root colonization increased in both treatments (i.e. AMF and AMF-T) over time (Fig. 18). A significant increase of AM fungal potato root colonisation was observed in the AMF-T treatment as compared with the AMF treatment at weeks 12 and 16, with values ranging from 14.5 ± 5.1% in the AMF treatment to 18.9 ± 5.4% in the AMF-T treatment at weeks 12; and from 22.7 ± 4.9% in the AMF treatment to 30.1 ± 5.1% in the AMF-T treatment at weeks 16. At weeks 20, no significant difference was observed between the AMF and AMF-T treatment. No differences in arbuscules and vesicles colonization were observed between both treatments (Fig. 18).

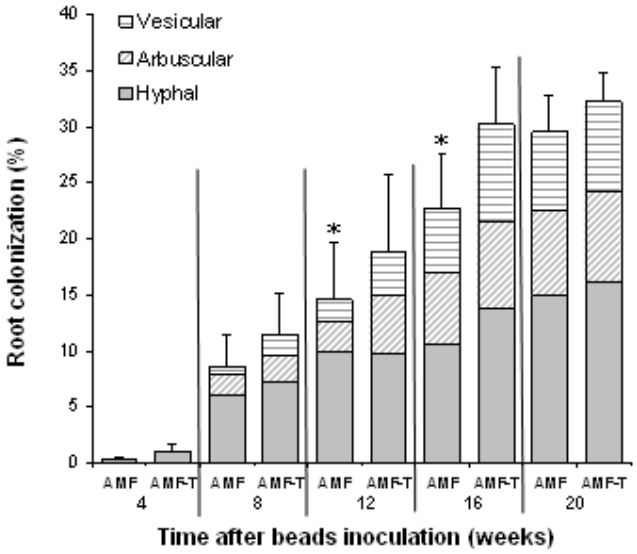

Figure 18 Influence of *T. harzianum* on the percentage of AM root colonization (hyphal, arbuscules and vesicles) of autotrophic potato plantlets (*S. tuberosum*). AMF represents the AM fungus alone, AMF-T represents the AM fungus associated with *T. harzianum*. Data represent means of five replicates. The bars indicate standard deviations of total root colonization. Column followed by * differed significantly between treatments at $p \leq 0.05$ (Tukey test).

For both treatments (i.e. AMF and AMF-T), the evolution of the MF followed the same pattern (Fig. 19). The values increased during the first 12 weeks (0.32 ± 0.03 in the AMF treatment and 0.45 ± 0.02 in the AMF-T treatment) and slowed down from week 12 to week 20 (0.24 ± 0.01 in the AMF treatment and 0.30 ± 0.06 in the AMF-T treatment at week 20).

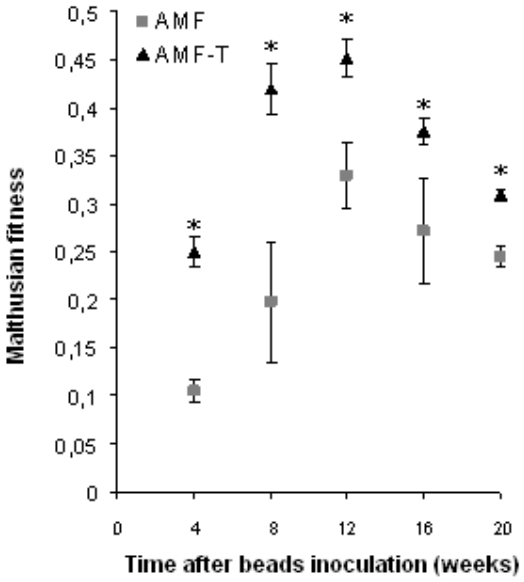

Figure 19 Malthusian fitness measured on the sporulation dynamics of the AM fungus in presence (AMF-T treatment) or in absence (AMF-treatment) of *T. harzianum.* AMF represents the AM fungus alone, AMF-T represents the AM fungus associated with *T. harzianum*. Data represent means of ten replicates (mean ± standard deviations). Values followed by * differed significantly between treatments at $p \leq 0.05$ (Tukey test).

Spores produced in presence/absence of *T. harzianum* were viable (Table 9). Germination of the spores was observed two days after plating on the MSR medium. The dynamics of spore germination did not differ between the spores sampled from the AMF or AMF-T treatments or in the AMF (+T) treatment (Table 9). Moreover, whatever the treatment considered (i.e. AMF, AMF-T and AMF (+T) treatments), the spores had the same colonization potential.

Table 9 Percentage of germination of the AM fungus in presence (AMF-T treatment) or in absence (AMF-treatment) of *T. harzianum*, measured 3, 7 and 11 days following entrapment in alginate beads.

Time (days)	3	7	11
AMF	78.0±15.7a	90.0±9.9a	94.0±7.1a
AMF-T	78.0±6.4a	86.0±7.9a	90.0±5.8a
AMF (+T)	86.0±9.3a	92.0±4.3a	96.0±3.6a

Data represent means of fifty replicates (mean ± standard deviations). For each time of observation (*i.e.* 3, 7 and 11 days following entrapment), values in the same column followed by identical letter did not differ significantly ($p \leq 0.05$, Tukey test).

The first contact between the AM fungal hyphae and the roots were observed within 15 days. After 6 weeks, typical AM extraradical structure were observed in both treatment and an average of 158 ± 69 spores were produced in the AMF treatment and ranged from 284 ± 85 spores in the AMF-T treatment to 234 ± 93 spores in the AMF (+T) treatment. At that time, the total root colonization ranged from 3.8 ± 2.6% in the AMF treatment to 5.2 ± 1.9% in AMF-T treatments and 5.1 ± 2.3% in AMF (+T) treatment.

6. Discussion

The application of beneficial microorganisms is nowadays encouraged in agriculture because of their potential to improve plant diseases resistance and to increase crop production in an environmentally-friendly way. Inoculum of AM fungi and *Trichoderma* sp. are available commercially in a variety of forms ranging from wettable powders and granules to potting media (reviewed in Kaewchai *et al.*, 2009). Among the carriers, alginate beads have been extensively used in laboratory studies for inoculum formulation and are compatible with industrial scaling-up (Bashan, 1998; Kaewchai *et al.*, 2009). One important advantage for immobilized microbes is the good storage capacity for practical applications (Sanyal *et al.*, 2003; Plenchette and Strullu, 2003). A number of studies have reported the germination and re-growth of spores of *Glomus* sp. and conidia of *T. harzianum* entrapped individually in alginate beads (Declerck *et al.*, 1996b; Fravel *et al.*, 1985) but none, to our knowledge, investigated the co-entrapment of both fungi. Here we demonstrated that, the co-entrapment of both microorganisms did not hinder germination and re-growth of *Glomus* sp. or *T. harzianum.* The saprotroph did not affect the AM symbiosis establishment but rather stimulate the extra and intraradical AM fungal development and fitness.

In our study, the use of alginate beads as a carrier for the entrapment of *in vitro* produced spores of AM fungi and conidia of *T. harzianum* resulted in a high infective potential of both fungi

entrapped individually as well as in combination. However, a significantly higher %PIB was noted with *T. harzianum* as compared to *Glomus* sp. in the early days following entrapment (i.e. days 2 to 6). This could be attributed to the higher velocity of germination and mycelium growth rate of *T. harzianum*. Indeed, from earlier studies conducted by De Jaeger *et al.* (2010a, b) with *T. harzianum* and by Giovanetti (2002) with various species of AM fungi, it was shown that during the early phase of growth (i.e. 24h after conidia or spore incubation), *T. harzianum* grew between 1.64 and 10 time faster than the AM fungi germ tubes. As a result, *T. harzianum* crossed first the alginate coating.

For the establishment of the AM symbiosis, the first key events in the life cycle of AM fungi are propagule (i.e. spore in our case) germination, germ tube elongation and contact with the root surface of a mycotrophic plant. During these first events (grouped in the pre-symbiotic phase of AM fungal development), AM fungi are particularly sensitive to the presence of other microorganisms (Jansa and Gryndler, 2010). Synergistic as well as antagonistic interactions have been reported to impact the establishment of the AM symbiosis (McAllister *et al.*, 1994a, b; Fracchia *et al.* 1998; Martinez *et al.*, 2004). In the last decade, several studies have demonstrated the role of soluble exudates and volatile substances produced by *Trichoderma* species to stimulate or inhibit the development of the AM fungal pre-symbiotic phase (McAllister *et al.*, 1994a, Fracchia *et al.*, 1998, 2004; Martinez *et al.*, 2004). Lee and Koske, (1994) and De Jaeger *et al.*

(2010a) further suggested that the mycoparasitic nature of *Trichoderma* sp. may represent an important component of the decline in AM fungal spore viability. Interestingly, in our experiment, no mycoparasitism of *T. harzianum* was noted on the spores of *Glomus* sp. in the alginate beads, suggesting their compatibility to co-exist in a close confined environment. As synergetic, antagonistic or neutral relationships between both fungi have all been reported (Gryndler, 2000), it was suggested that variation in the interaction between AM and *Trichoderma* sp. during the AM pre-symbiotic phase may be due to differences between fungal species involved.

At present, little is known on the impact of *T. harzianum* on the AM effectiveness, measured as the extent of fungal development and metabolic activity of the intra or extraradical fungal structures (Tisserant *et al.*, 1993; Guillemin *et al.*, 1995). This might be because most studies were conducted under greenhouse and field conditions and have addressed the plant performance (Datnoff *et al.*, 1995; Chandanie *et al.*, 2009) rather than the interaction between both fungi. In our study, the presence of *T. harzianum* did not suppress, inhibit or impede the establishment of the mycorrhizal symbiosis. The potato roots were colonized within 4 weeks. This contradict earlier studies reporting that *Trichoderma sp*. was able to decrease the AM root colonization when the saprotroph was inoculated before or at the same time to the AM fungi (McAllister *et al.*, 1994b). The decrease of AM root colonization was not attributed to a direct effect of *T. harzianum* on the intraradical mycelium but to a negative effect on the AM pre-

symbiotic development of the AM fungus (McAllister *et al.* 1994b), a mechanism that was not observed in our experiment.

Our results demonstrated a significant increase of AM intra and extraradical mycelium length, in presence of *T. harzianum.* The mechanisms behind this observation remain unclear but could potentially be attributed to exudates produced by the saprotroph as demonstrated by Fracchia *et al.* (2004). These authors reported an increase in the AM extra and/or intraradical fungal development in presence of exudates produced by *Trichoderma* sp. The development of the intraradical mycelium was modulated by the effects of the saprotrophic fungi on the extraradical phase of the AM fungi. Similar observations was made by Green *et al.* (1999) who demonstrated that the presence of *T. harzianum*, restricted to a root-free compartment, was able to modulate the development of the intraradical phase of *Glomus* sp. in cucumber roots. According to these studies, communication between the different parts of the AM mycelium was hypothesized (Hodge *et al.*, 2010). Interestingly, Duffy *et al.* (2003) suggested that spore production was a stress response against parasitism. Similar results were obtained in presence of Collembola, grazing on the AM mycelium (Hanlon, 1981). This author demonstrated that low level of predation could stimulate the production of mycelium and spores as a stress reaction. In our study, visual evidence of coiling of *T. harzianum* around AM host hyphae and subsequent penetration of the extraradical mycelium supported this hypothesis.

The use of SDH has been proposed as a good method to evaluate the effects of biotic or abiotic factors on the AM fungal viability (Tisserant *et al.,* 1993; Guillemin *et al.,* 1995). The very limited decrease of AM fungal viability observed in our study contrasted with earlier results reported by De Jaeger *et al.* (2010a). These authors demonstrated, under *in vitro* culture conditions and with the same isolates, a significant decrease of the AM fungal viability due to the presence of *T. harzianum* when the saprotrophic fungus was inoculated on an established AM fungal colony. As previously demonstrated, competition for nutrient (i.e. carbon and nitrogen) and space is an antagonistic mechanism of *T. harzianum* (Harman *et al.*, 2004). Moreover, mechanisms related to mycoparasitism are strongly induced by the sensing of the host (Rincon *et al.*, 2009) and/or when a poorly assimilable carbon or nitrogen source is available (Rincon *et al.*, 2009). Consequently, it was suggested that the mycoparasitism process was expressed when *T. harzianum* was inoculated on an established AM network. In contrast, in our experimental study, the inoculation at the same time coupled with the fast mycelium development of *T. harzianum* allowed the saprotrophic fungus to access the resources and space before the AM fungus. As *T. harzianum* colonized the substrate prior to the invasion by the extraradical mycelium of *Glomus* sp., it was proposed that the absence of the host de-repressed genes encoding for the parasitism. According to these hypotheses, it was suggested that the antagonistic effect of *T. harzianum* on the AM mycelium viability (i.e. SDH staining) may be

decreased when the saprotrophic fungus was co-entrapped and inoculated at the same time as *Glomus* sp.

Interestingly, our results suggested that *T. harzianum* increased the reproductive activity of *Glomus sp.* as demonstrated by the Malthusian fitness measure. The newly produced spores were able to germinate, colonize a new host and reproduce the fungal life cycle. Consequently, as spores represent a main source of inoculum in soil (Smith and Read, 2008), the presence of *T. harzianum* in mixed inoculum may represent an adequate mean to increase the inoculum potential of AM fungi in field.

In this study, we demonstrated that the co-entrapment represents a promising formulation for the development of inoculants combining AM fungi with *T. harzianum.* Our results clearly confirmed the capacity of both fungi in combination to re-growth outside the calcium-alginate coating and to colonize a susceptible plant. This corroborates earlier findings combining cells of the P-solubilizing yeast *Yarrowia lipolytica* or *Bradyrhizobium sp.*, a plant growth promoting rhizobacteria with AM fungi (Vassilev *et al.*, 2001; Weber *et al.*, 2005). Our study demonstrated a weak mycoparasitism effect of *T. harzianum* that did not impact the AM fungal development but rather stimulate spore production. This study, is the first that clearly demonstrate that the presence of *T. harzianum* modulates the fitness of an AM fungus. Even though the *in vitro* system is useful to investigate AM fungal-saprotrophic fungal interactions, the

extrapolation of these results to field conditions should be done to test the reliability of this formulation under field conditions and to understand how beneficial microorganisms interact with their plant host or respond to their environment.

Acknowledgements This research was sponsored by the Direction générale opérationnelle de l'Agriculture, des Ressources naturelles et de l'Environnement du service public de Wallonie under contract number D31-1193, entitled « Valorisation de la microflore bénéfique des sols pour le contrôle de la flore pathogène des productions de pomme de terre comme alternative à l'utilisation des pesticides ».

IX. GENERAL DISCUSSION

Growing awareness on the environmental damage caused by chemicals used for the control of plant disease in modern agriculture has raised the need for biological alternative approaches. Among these, the use of beneficial microorganisms improving plant health and productivity and protecting the environment has received increased interest. Many soilborne microorganisms have been described, characterized, and tested for their use as plant growth promoters and biocontrol agents and are nowadays considered in integrated pest and productivity management practices (Antoun and Prevost, 2005).

In non-perturbed soil systems, the rhizosphere represents a highly dynamic region governed by a complex mosaic of interactions between plants and microorganisms (Kennedy and De Luna, 2004). AM fungi interact with a wide range of other microorganisms at all stages of their life cycle and these microbial communities tend to live in relative harmony where all population generally balance each other in their quest for food and space (Bélanger and Avis, 2002). In arable soil (*i.e.* perturbed soil systems), crops are removed at the end of the growing season before re-sowing of another crop. Under such practice, the AM fungi continually have to re-establish themselves (Hodge, 2000). Other agricultural practices (*i.e.* pesticides and fertilizer usage) also impact the natural balance of soilborne microorganisms which alter the microbial community structure and can lead to the loss of beneficial microbes (Avis *et al.*, 2008). In these situations, the rehabilitation (via inoculation) of beneficial

microorganisms into productions systems appears a sustainable and necessary practice. As a prerequisite to the application of microorganisms, it is essential to ascertain their compatibility (or absence of antagonism) as well as their competitive potential with the native microflora.

T. harzianum is nowadays broadly used for the control of undesirable fungal plant pathogens in sustainable agriculture. Identically, AM fungi are among the most widely used microorganisms for plant growth promotion and control of root pathogens. The beneficial combination of both microbes has been observed in several studies (Calvet *et al.*, 1993; Linderman, 1994; Datnoff *et al.*, 1995; Chandanie *et al.*, 2005, 2009), but a number of studies also report the reverse, emphasizing a side-impact of *T. harzianum* on the non-target AM fungus (Rousseau *et al.*, 1996; De Jaeger *et al.*, 2010a). AM mycelium represents a considerable biomass into the soil environment (Miller *et al.*, 1995; Olsson *et al.*, 1999) and may therefore be affected by the presence of mycoparasitic microorganisms (Purin and Rillig, 2008) in a competitive environment.

Using an experimental strictly controlled microcosm with potato plants, we investigated the interaction between *T. harzianum* MUCL 27909 and *Glomus* sp. MUCL 41833. We demonstrated that the presence of the saprotrophic fungus impacted the AM fungal viability (**Chapter 3**) and functionality (**Chapter 4**) when it was

inoculated on an established network of *Glomus* sp. The mycoparasitism was observed both on the extra- and intraradical hyphae and spores suggesting that the saprotrophic fungus used the cell walls and cytoplasmic contents as nutritional resources (Benhamou and Chet, 1997). Mycoparasitism could thus represent a mechanism of control of the AM fungus by *T. harzianum*, which does not exclude the potential involvement of other mechanisms (*i.e.* competition and antibiotics production) allowing *Trichoderma* species to be antagonists on AM fungi.

As demonstrated in **Chapter 1**, *T. harzianum* MUCL 29707 is unable to penetrate the roots of potato plantlets grown *in vitro* (Gallou *et al.*, 2010). Consequently, it was suggested that the presence of *T. harzianum* within the potato roots was exclusively related to its entry via the parasitism of AM extraradical hyphae (**Chapter 3**). The coenocytic nature (*i.e.* the absence of septa in living hyphae) of the AM mycelium linked to the protoplasmic continuity between the extra- and the intraradical phase of AM fungi (Smith and Read, 2008) may facilitate this process and represent a unique pathway for the entry of *T. harzianum* into the roots. This observation opens the debate on the susceptibility of AM fungi of being infected by microorganisms (*i.e.* pathogens or biological control agents) from the rhizosphere and to act as a channel for root penetration.

As previously demonstrated, some *Trichoderma* strains are able to colonize root surfaces (Yedidia *et al.*, 1999; Harman *et al.*,

2004; Chacon *et al.*, 2007); but through chemical communication factors they induce the plant to get rid of the invading *Trichoderma* hypha so that the saptrotrophic fungi is restricted to the outer layer of the root (*i.e.* epidermis) (Yedidia *et al.*, 1999). So far, only the study of Evans *et al.* (2003) has reported the colonization of the vascular system of cocoa by these fungi (*i.e. T. harzianum, T. hamatum, T. virens, T. viride* and four undescribed species). By this colonization, *Trichoderma* spp. induces localized resistance to plant pathogen attack, as well as systemic interaction within the plant (Yedidia *et al.*, 1999, 2003; Harman *et al.*, 2004; Shoresh and Harman, 2008). A recent study showed that *T. harzianum* strain 22 (T-22) interaction with maize plant roots resulted in controlled activation of the carbohydrate metabolic process as well as enhancement of photosynthesis, providing the growing plant with more energy and carbon source for their growth (Shoresh and Harman, 2008). According to these observations, the presence of *Trichoderma* spp. inside the root did not have detrimental effect on the plant (**Chapter 3**). Up-regulation of carbohydrate metabolism and resistance responses may correspond to the enhanced growth response and induced resistance, respectively, conferred by the *Trichoderma* root colonization (Shoresh and Harman, 2008).

The disruption of the AM mycelium network by the mycoparasite *T. harzianum* has impacted the capacity of the AM fungus to transport P (**Chapter 4**) and to acquire C from the plant host via a reduced viability and metabolic activity noted on the hyphae. By

disrupting the continuity between extraradical hyphal sections and intraradical mycelium, the saprotrophic fungus may have important effects on the functioning of the AM fungus and the plant performance indirectly by decreasing the source-sink relationship between the AM fungus and its host plant. As suggested, the AM intraradical structure would still receive carbon compounds from the plant host but the reciprocal transaction, *i.e.* the transport of nutrient to the roots, would be prevented (Fitter and Sanders, 1992; Gange, 2000). In this situation, the relationship between AM fungi and their host plant would become parasitic rather than mutualistic (Johnson *et al.*, 1997), a condition that causes reduction of plant growth and yield (Klironomos, 2003; Lendzemo *et al.*, 2004).

Trichoderma species are considered necrotrophic parasite of filamentous fungi, having a wide host range and less-specific mode of action (Jeffries, 1995; Viterbo *et al.*, 2007; Viterbo and Horwitz, 2010). However, there is no clear evidence of necrotrophic or biotrophic parasitism in AM fungi (Purin and Rillig, 2008). In established AM mycelium networks, we observed hyphal invasion, rapid AM cytoplasm degradation followed by dead of the hyphae and spores viability loss (**Chapter 3**) supporting the hypothesis that *T. harzianum* is an invasive necrotrophs (Jeffries, 1995). Conversely in **Chapter 5**, both organisms were inoculated at the same time and the impact of *T. harzianum* on the AM fungus more closely resembled biotrophic mycoparasitism. The growth of the AM fungus, its ability to sporulate and general metabolism seemed not affected, supporting

earlier findings (Jeffries, 1995; Viterbo *et al.*, 2007; Viterbo and Horwitz, 2010). According to these observations, it is suggested that there is a continuum of parasitic behavior of *T. harzianum* on AM fungi ranging from biotrophic to necrotrophic.

AM fungi, as other fungi, have developed a range of mechanisms to resist antagonism by saprotrophic fungi. Paulitz and Menge (1984) suggested that spores exude substances that act as repellants, preventing saprotrophic fungi to grow on healthy AM fungal spores. Purin and Rillig (2007) further suggested that glomalin, a glycoprotein produced by AM fungi, may confer resistance against parasitism. The degree of melanization of the spore wall was also reported to confer resistance to mycoparasitism (Sneh *et al.*, 1977; Daniels and Menge, 1980). White or light-colored spores (*e.g. G. macrocarpum* or *Gi. candida*) are more susceptible to parasitism than dark-colored spores (*e.g. G. contrictum* or *Gi.giganta*) (Ross and Ruttencutter, 1977; Schenck and Nicholson, 1977; Sheh *et al.*, 1977; Bhattacharjee *et al.*, 1982). Finally, septum formation was suggested as a mechanism to isolate damaged sections in the AM mycelium caused by physical stresses (de Souza and Declerck, 2003; de la Providencia *et al.*, 2005). This mechanism of resistance to fungal invasion was hypothesized in **Chapter 3** but no septum formation was observed close to the appressoria formed by *T. harzianum* on the AM hyphae surface or following hyphae invasion and no significant differences were observed in the number of hyphae with septa between treatment with and without *T. harzianum*. This suggested that

the mechanism of isolation of hyphal segments frequently reported in the AM fungus to restrict cytoplasm leakage under physical stress situations (de la Providencia *et al.*, 2005) was not operational under *T. harzianum* pressure. One may hypothesize that the rapid invasion by *T. harzianum* preceded the formation of septa. Moreover, it was suggested that AM septum was not an efficient barrier to prevent the saprotrophic fungus to develop within the AM fungal hyphae (**Chapter 3**).

Many examples of mycoparasitism, including on AM hyphae, have been reported under laboratory conditions. However, these studies should be taken with caution and extended to soil environments before generalization of the phenomenon. Mycoparasitism was reported on fossil of AM fungal spores from the Lower Devonian Rhynie chert (400 million years old) suggesting an early phenomenon in evolution (Hass *et al.*, 1994). According to these observations, it was suggested that mycoparasitism has co-evolved with mycorrhizal fungi (Jeffries, 1995). Some evidences from field work showed that a large number of AM fungal spores were parasitized (Malençon, 1930; Lee and Koske, 1994). The presence of many fine radial canals (*i.e.* penetration canals) in the wall of AM spores (Malençon, 1930; Jeffries *et al.*, 1988) and the isolation of potential fungal parasites inside perforated spores (Lee and Koske, 1994) supported this hypothesis. Several studies demonstrated that parasitized AM spores have been healthy at the time of recognition, attachment and penetration of the host by the mycoparasite (Jeffries

and Young, 1978; Lee and Koske, 1994), thereby influencing dynamics of fungal communities (Lee and Koske, 1994) and reducing their inoculums potential in the field (Paulitz and Menge, 1986; Lee and Koske, 1994). Paulitz and Menge (1986) demonstrated that mycoparasitism activity thus indirectly affected the crop yield as the rate of root colonization was reduced.

In contrast to spore mycoparasitism, no study demonstrated the parasitism of hyphae under field condition. Under laboratory controlled conditions, interactions studies were often conducted with pure mixed culture under adequate environmental condition and nutrient excess, while under field condition microbial competition may evolve under more harsh environmental conditions, favorable to competition via antagonistic relationships (Jeffries, 1995). Several studies demonstrated that the parasitism behavior of *Trichoderma* sp. is expressed under nutrient-scarce environment (*i.e.* under depleted carbon or nitrogen sources) (Benitez *et al.*, 1998; Lorito *et al.*, 1998). In this respect, the ability to parasitize and utilize other fungi as nutrient source must confer an advantage, and thus it is likely that mycoparasitism occurs more widely than is currently reported (Jeffries, 1995).

On the other hand, under *in vitro* conditions, studies are generally set up with a single plant-AM fungus-beneficial microorganism considering a small number of plant and fungal taxa, and with little or no attention paid to the other soil biota with which

microorganisms must interact (Hodge, 2000). Consequently, the unique source to feed for the mycoparasite is the AM fungus. Then at reasonably high density we would expect to see a detrimental effect on the functioning of the symbiosis (**Chapter 4**). Although there are no doubts that *Trichoderma* sp. are capable of parasitizing mycorrhizal hyphae, in presence of fungal diversity it remain to be tested if *Trichoderma* sp. will consistently parasitize on AM mycelium in preference to other fungi and thus will decrease the functionality of the AM symbiosis.

The significance of mycoparasitism in natural environments is certainly underrated, possibly because of the difficulty in making field observations (Jeffries, 1995). By parasitizing the extra and intraradical mycelium, the parasite might potentially reduce the ability of mycorrhizal fungi to supply limiting soil resources to its host (Warnock *et al.*, 1982; Fitter and Sanders, 1992; Fitter and Garbaye, 1994) and might shift the mycorrhizal symbiosis even futher toward parasitism (Johnson *et al.*, 1997), and consequently causes reduction of plant growth and yield (Klironomos, 2003; Lendzemo *et al.*, 2004).

In natural condition, Handelsman and Stabb (1996) suggested that antagonistic microorganism are thought to exert only limited pressure on the host since they operate in microsites where only a fraction of the target community is exposed during a short period of its life cycle. Thus, it was expected that parasitism exert a relatively low pressure on host population. Nevertheless, if only some AM fungi

species in a resident community are affected by parasitism, this could lead to the relative predominance of AM fungi species with greater resistance (Purin and Rillig, 2008) *e.g.* interfungal parasitic relationships are likely to play an important role in the development of AM fungi community structure (Jeffries, 1995). Consequently, changes in AM fungal communities may impact the plant growth (Maherali and Klironomos, 2007) and plant community composition (van der Heijden *et al.*, 1998).

In cultural systems, the parasitic effects on AM fungal inoculum may reduce the benefit conferred to plants, with considerable economic consequences, as AM fungi inoculum production is a growing worldwide industry (Gianinazzi and Vosatka, 2004; Schwatz *et al.*, 2006). In sustainable agriculture, the application of biocontrol agents such as *T. harzianum* that may impact the non target AM fungi, may result in a disadvantage for the host plant due to decreased mycorrhizal efficiency (Purin and Rillig, 2008).

The fact that *Trichoderma* sp. is a parasite of AM fungi is not entirely supported by the literature. According to the results obtained in **Chapter 5,** numerous studies reported an absence of antagonism of *Trichoderma* sp. on AM fungi (Paulitz and Linderman, 1991; Green *et al.*, 1999; Vazquez *et al.*, 2000). Moreover, some authors demonstrated a synergetic interaction between *Trichoderma* sp. and AM fungi (Calvet *et al.*, 1993; Chandanie *et al.*, 2009). Beneficial effects of the introduction of combination of these microorganisms on

plant growth have been reported for numerous crops including citrus, cucumber, tomato (Camprubi *et al.*, 1995; Chandanie *et al.*, 2009; Srivastava *et al.*, 2010). Consequently, the combined effects of these interactions may be exploited for the benefit of sustainable agriculture.

Producing microbial inoculants for agriculture is a complex procedure that involves not only the development of the necessary biotechnological expertise, but also the ability to respond to the specifically related legal, ethical, educational, and commercial requirements (Gianinazzi and Vosatka, 2004). Inoculants have to be designed to provide a dependable source of beneficial microorganisms in an adequate carrier that could be stored without losing viability and distributed under a wide range of field condition. In recent years the method of immobilizing living cells has addressed a wide range of applications (Cassidy *et al.*, 1996; Bashan, 1998). Entrapment of microbial cells for soil application provides a range of advantages such as ease of application in the soil, reduced off-site drifting, (Vassilev *et al.* 2001b) and protection of cells from environmental stress including antagonistic fungi (**Chapter 5**). Results obtained in **Chapter 5** have confirmed the effectiveness of the co-entrapment process to be use as potential inoculant, as reported for different AM-microbial combinations (Vassilev *et al.*, 2001b; Weber *et al.*, 2005) or plant growth-promoting bacteria-*Trichoderma* (El-Katatny, 2010). As mixed inoculation of biocontrol microorganisms is more efficient in controlling pathogens than the use of single-strain inoculants, the inclusion of other beneficial microorganisms including phosphate-

solubilizing bacteria or/and plant growth-promoting rhizobacteria with the AM fungi and *Trichoderma* sp. in alginate beads could be beneficial to different applications in the fields. Nevertheless, a prerequisite for the successful and consistent combination of beneficial microorganisms is to ascertain the compatibility of the inoculated microorganisms. Moreover it is important to evaluate the compatibility of the microbial inoculants with the target environment (Estaún *et al.* 2002; Vosátka and Dodd 2002; Requena *et al.* 1996).

X. CONCLUSIONS

Interactions between *Trichoderma* sp. and AM fungi have not been extensively explored so far, partly due to the difficulty to grow both organisms under rigorous controlled conditions (*e.g. in vitro*).

Here we investigated the impact of *T. harzianum* on an AM fungus and its consequent effects on the AM fungal physiology. Three central questions were addressed:

1. Is it possible to extend the classical *in vitro* culture systems used with single microbes, to multiple microbes (i.e AM fungi and *T. harzianum*)?

The autotrophic *in vitro* culture system (HAM-P) developed by Voets *et al.* (2005) is an important tools to study plant-fungi interactions, either obligate symbiont (*i.e. Glomus* sp.), non-obligate plant-beneficial fungi (*T. harzianum*) or pathogenic (*R. Solani*). This system present several advantages, among which are the presence of a photosynthetic plant with a normal hormonal balance and physiological source-sink relationships (Fortin *et al.*, 2002) and the absence of undesirable microorganisms. Moreover, the use of synthetic medium allows direct observation of the fungal development and interactions with the plant. An adaptation of the HAM-P *in vitro* culture system allowed a fast and homogenous mycorrhization of seedlings within a few days by using the symbiotic phase of the fungus as inoculum (Voets *et al.*, 2009).

The ability to couple and to integrate the *in vitro* culture systems with different tools (*i.e.* cryo-SEM analysis (**Chapter 1**), gene expression analysis (**Chapter 2**)) opens the door to increase our understanding on how soilborne microorganisms and plants behave, including gene expression during their interactions, giving a more complete picture of the plants-fungal cells interaction.

2. How do *T. harzianum* affect the AM symbiosis?

It was demonstrated in **Chapter 3** that *T. harzianum* mycoparasitized *Glomus* sp. when it was inoculated on AM established mycelium networks. This phenomenon occurred through a multistep dynamic invasion process of the ERM (*i.e.* extraradical hyphae and spores) involving contact, surface attachment and penetration, and its subsequent extension into the IRM (*i.e.* intraradical hyphae and vesicles) before exiting in the root cells. Intrahyphal growth of *T. harzianum* was followed by a rapid degradation of the AM cytoplasmic content, decreasing viability (**Chapter 3** and **4**) and metabolic activity (**Chapter 4**) of AM fungal hyphae. By disrupting the AM mycelium network, the mycoparasite *T. harzianum* impacted the capacity of the AM fungus to transport P (**Chapter 4**). Taken together, this suggested that *T. harzianum* could impede a functional symbiosis. In addition, as demonstrated in **Chapter 3**, the ERM of the AM fungus

represented a pathway for the entry of *T. harzianum* into the roots of potato. This observation opens the debate on the susceptibility of AM fungi of being infected by microorganisms and to act as a channel for root penetration.

3. Does co-entrapment of *Glomus* sp. with *T. harzianum* fungi within the same alginate inoculum affect the germination and establishment of the AM symbiosis?

Alginate co-entrapment process represents a promising approach for the development of microbial inoculants containing both AM fungi and *T. harzianum*. Our results (**Chapter 5**) demonstrated that co-entrapment did not modify the inoculum potential of each microorganism. Entrapment within beads offers an excellent protection of AM spores against many biotic and abiotic environmental constraints including the anatogonistic fungi, *T. harzianum*. As *T. harzianum* increased the AM inoculums potential (*i.e.* spores), dual inoculation with an AM fungus and *T. harzianum* co-entrapped in alginate may represent an interesting inoculant for field application.

The largest part of knowledge on AM symbiosis derives from microcosm experiments using a small number of plant and fungal taxa, and with little or no attention paid to the other soil biota with which they come into contact. The purpose of this thesis was to focus

on the interactions between the AM fungus and the saprotrophic *T. harzianum* on potato plants and the implication of these interactions for mycorrhizal development and functioning in ordre to develop an inoculums formulation. We clearly demonstrated that *T. harzianum* infected the AM fungus affecting the viability and the functionality of the symbiont. Howevers, when both fungi were co-entrapped in the same alginate bead, the alginate coat conferred a protection to the AM fungal spores and limited mycoparasitism was noted. Under these conditions, we observed a promotion effect of the saprotrophic fungi on the development of *Glomus* sp. which may represent an added-value for the application of both microorganisms. Co-entrapment in alginate beads of an AM fungus with *T. harzianum* may represent an interesting inoculum for application in agro-environments.

XI. PERSPECTIVES

As stated by Gange (2000), there is an intriguing paradox in AM fungal ecology. Although numerous laboratory studies have demonstrated a vast panel of responses in microorganisms-AM fungal interactions, only occasionally were these observations transposed to natural situations. It is thus necessary to combine fundamental approaches under strict-controlled conditions with *in situ* observations to ascertain that the mechanisms prevailing under controlled culture conditions are similar in the field and have ecological meaning and applicability.

Given the complexity of the soil system, it is necessary to design microcosm experimental systems that are ecologically realistic. A number of conditions should be met to conclusively demonstrate the occurrence and impact of parasitism on AM fungi in field condition. This could be partly achieved with the HAM-P *in vitro* culture system used in our study combining a photosynthetic active plant grown on a sugar-free medium with and AM fungus, *Glomus* sp. and a saprotrophic fungi, *T. harzianum*. With this system, the mechanisms of mycoparasitism could be extended to complex and interaction-rich associations with ecologically realistic combinations of AM fungi and non AM fungi.

For instance, in the last decade, a number of studies have separated the AM fungi in r and K strategists. The K-strategists are organisms that evolved traits to enhance survival in stable and predictable environments where competition is high. K-strategists

principally allocate resources to growth and enhanced survival. To the contrary, the r-strategists invest their energy mainly in the production of many offspring, and evolved traits that are favoured in instable environments. Their survival strategy differs markedly via, among other, different hyphal healing mechanisms developed to resist stress situations. The interaction of a population of r and K strategists, an obvious situation in soil, with *T. harzianum* would thus yield important results on the dynamics of AM fungal populations both in natural (non-perturbed) conditions and in systems close to agro-environments. The HAM-P systems offer a unique possibility to investigate such complex interactions and further to understand the impact of multi-faceted AM fungi on nutrient (*e.g.* P) transport.

An additional challenge is to identify the AM fungi life history stage that is the most prone to be infected by the mycoparasite and to evaluate the effects of such phenomenon. If spore are parasitized, the question about the establishment of the symbiosis and dispersal of the species may be addressed. If AM mycelium is affected, it will be necessary to evaluate the effect of the disruption of the network on the AM symbiotic efficiency and plant community.

An alternative view is that AM fungi are not highly susceptible to mycoparasites under field condition. If such is the case, it will be necessary to study the defence strategies that confer apparent resistance to parasites in a high-level interactive environment like rhizosphere. Based on the mechanisms developed by plant pathogens

in response to antagonism, we could focus on the production of secondary metabolites by the AM mycelium (*i.e.* melanin, glomalin), and on the deposit of compounds (*i.e.* crystals or others) on the hyphal surface.

Further questions should also be addressed regarding the role of AM fungi as a pathway for the entrance of *T. harzianum* into the roots of potato and by extension other plants. This mechanism was demonstrated in our work but its ecological significance remains to be evaluated. This observation also opens the debate on the susceptibility of the AM fungi to be infected by other microorganisms from the rhizosphere.

Finally, the AM fungus seems not affected by the presence of parasite microorganism like *T. harzianum* in its asymbiotic phase when it was entrapped in alginate bead. Thus, the combination of both beneficial organisms may represent an alternative to pesticides (and fertilizers) in agro-environments. A key difference between chemical and biological control is that antagonistic microorganisms are capable to adapt their responses to changes in pathogen populations. Co-evolution favor the equilibrium between organisms where the level of control remains relatively stable (Holt and Hochberg, 1997). In this respect, the development and testing of consortia of microorganisms exhibiting multiple beneficial and complementary mechanisms of action seems a realistic strategy to control pathogens and thus may

represent a sustainable alternative or complementary approach to chemical uses under field condition.

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XV. OVERVIEW OF THE SCIENTIFIC ACHIEVEMENT

Articles

1. **De Jaeger N**, Declerck S, de la Providencia IE. 2010. Mycoparasitism of arbuscular mycorrhizal fungi: a pathway for the entry of saprotrophic fungi into roots. FEMS Microbiology Ecology 73: 312-322.
2. **De Jaeger N**, Decock C, Declerck S, de la Providencia IE. 2010. Coupling autotrophic *in vitro* plant cultivation system to scanning electron microscope to study plant-fungal interactions. Spanish Journal of Agricultural Research 8: S69-S78.
3. Gallou A, **De Jaeger N**, Cranenbrouck S, Declerck S. 2010. Fast track *in vitro* mycorrhization of potato plantlets allow studies on gene expression dynamics Mycorrhiza 20: 201-207.

Posters

1. **De Jaeger N**, Declerck S and de la Providencia IE. Mycoparasitism of arbuscular mycorrhizal fungi: a pathway for the entrance of saprophytic fungi into the roots. 6th international conference on Mycorrhizeae (ICOM 6). Belo Horizonte (Brazil).
2. **De Jaeger N**, Dupré H, Declerck S, de la Providencia IE. Effet de *Trichoderma* sur la symbiose mycorhizienne. 2ème Journée francophone des Mycorhizes. Bruxelles (Belgique).

Teaching and student supervision

1. Manikak O. (Septembre 2009). Rôle du champignon mycorhizien à arbuscules dans le contrôle de la bactérie pathotène *Pectobacterium carotovorum* ssp. *carotovorum* (faculté des Sciences, Biologie, UCL).
2. Doat A. (Septembre 2010). Effet des champignons mycorhiziens à arbuscules sur la croissance et le rendement de 4 variétés de pommes de terre andines. (Faculté de sciences agronomiques et d'ingéniorie biologique, UCL)

Scientific trainings

1. International Training on *in vitro* Culture of Arbuscular Mycorrhizal Fungi: *Plant-systems* training. Louvain la Neuve, Belgium.
2. International Training on *in vitro* Culture of Arbuscular Mycorrhizal Fungi: *ROC system* training. Louvain la Neuve, Belgium.

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2009

N°148, Pokrzywa Wojcieh, Targeting of the yeast Sna3p and Sna4p to the endosomal pathway depends on their interaction with ubiquitin ligase Rsp5p (Promoteur: Morsomme P. / février 2009)

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