RESEARCH ARTICLE

Trichoderma harzianum might impact phosphorus transport by arbuscular mycorrhizal fungi

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Abstract

Trichoderma sp. is a biocontrol agent active against plant pathogens via mechanisms such as mycoparasitism. Recently, it was demonstrated that *Trichoderma 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 the 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 the presence of *T. harzianum*, possibly related to a stress reaction caused by mycoparasitism. In addition, the disruption of AM extraradical hyphae in the 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.

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 (Harman *et al.*, 2004; Whipps, 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 the presence as well as in the absence of plant pathogens (Datnoff *et al.*, 1995; Siddiqui & Mahmood, 1996; Chandanie *et al.*, 2009), while others 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 latter is the antagonistic nontarget action of *Trichoderma* spp. via mycoparasitism on the AM fungal mycelium (Lee & Koske, 1994; Rousseau *et al.*, 1996; De Jaeger *et al.*, 2010).

Phosphorus is required by plants in a relatively large amount, but is often poorly available. The low solubility and the slow diffusion of P in soil solution cause plants to become P deficient and to grow poorly (Smith & Read, 2008). To overcome this limitation, plants have developed several mechanisms of P acquisition, among which is the association with AM fungi (Hinsinger, 2001; Vance *et al.*, 2003). These microorganisms form symbiotic associations with nearly 80% of terrestrial plants (Smith & Read, 2008). This symbiosis is characterized by a reciprocal exchange of nutrients (principally P) for carbon (Smith & Read, 2008). To increase plant P acquisition, the extraradical mycelium (ERM) of AM fungi extends into the soil, increasing the absorptive area of roots significantly (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 cell to the plant cell (Cooper & Tinker, 1978; Jakobsen *et al.*, 1992; Smith & 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 in AM fungi in several studies (Lee & Koske, 1994; Rousseau *et al.*, 1996; De Jaeger *et al.*, 2010). Recently, De Jaeger *et al.* (2010) demonstrated that *Trichoderma 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 the 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).

Materials and methods

Potato plantlet stock

In vitro micro-propagated potato plantlets (*S. 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 ($90 \times 60 \times 50$ mm, 20 cuttings per box) containing 50 mL of Murashige–Skoog medium (Murashige & 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}.

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, MO) and adjusted to pH 5.5 before sterilization (121 °C for 15 min) (termed MSR^1). 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.

AM fungus 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 of carrot (*Daucus carota* L.). The cultures were provided in Petri plates (90 mm diameter) on MSR medium (see 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.

Trichoderma harzianum culture

A culture of *T. 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 a fresh PDA medium (25 mL) in a Petri plate (90 mm diameter). The Petri plate was subsequently incubated for 4 days in the dark at 25 °C and afterwards at 4 °C until needed.

Experimental set-up

A mycelium donor plant (MDP) *in vitro* culture system was set up for the fast and homogenous mycorrhization of 10-day-old potato plantlets following the methodology described by Voets *et al.* (2009). After 12 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 12.4×8.5 cm (each compartment 3.1×8.5 cm) (quadri-PERM, Greiner Bio-One, Kremsmünster, Austria) (Fig. 1). 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 (6.2×8.5 cm) was filled with 50 mL of MSR^1. In this compartment, one mycorrhized potato plantlet was placed with the roots on the MSR^1 medium and the shoots extending outside the culture plate. This compartment was

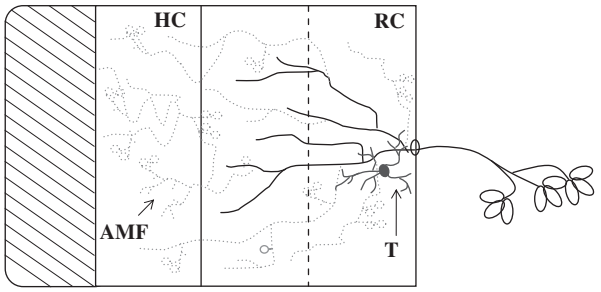

Fig. 1. Schematic representation of the quadri-compartmented culture system (plate 12.4 × 8.5 cm) used to study the impact of *Trichoderma harzianum* MUCL 29707 on the transport of ^{33}P by the AM fungus *Glomus* sp. MUCL 41833. Only two compartments were used. An enlarged root compartment (RC; 6.2 × 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 with the AM fungus in this compartment, while the ERM of the AM fungus was allowed to spread in a second compartment, named the hyphal compartment (HC; 3.1 × 8.5 cm). *Trichoderma 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.

referred to as the root compartment (RC). The other compartment was filled with 20 mL of MSR[1]. This compartment was referred to as the hyphal compartment (HC) and was only accessible to the ERM of the AM fungus. 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 millilitres of MSR[1] 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[1]. This favoured a homogenous regrowth of the ERM in the HC. After a period of 10 days, 40 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 × 6.7–40 magnification following the method of Declerck *et al.* (1996) and Giovannetti & 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 with the weight of a transparent plastic sheet representing the full HC. The number of spores was 3180 ± 151 and the length of the hyphae was 2633 ± 109 cm in the RC and 39 ± 29 spores and 1267 ± 129 cm in the HC covering 23 ± 4 cm^2 (91 ± 15%) of this compartment. The number of leaves of the potato plantlets was also evaluated to 6 ± 1 leaves.

The 40 culture systems were divided into two homogenous groups of 20 systems based on the above criteria. One group (named the AMF treatment) contained potato plantlets associated with the AM fungus. The other group (named the AMF-T treatment) also contained potato plantlets associated with 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. 1). The Petri plates were incubated horizontally in a growth chamber under the same conditions as above.

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

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, 10 Petri plates of each treatment (i.e. AMF and AMF-T treatments) were randomly selected among the two groups of 20 systems and a source of phosphorus (^{33}P) was added to the HCs. The source of ^{33}P was orthophosphate in diluted hydrochloric acid (< 0.1 M) supplied by PerkinElmer (Zaventem, Belgium). The radioactive solution was filter-sterilized (Acrodisc Syringe filters, PALL Corporation, Ann Arbor, MI) and 100 μL was applied with a micropipette in each system on the entire 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 four systems of each treatment, formaldehyde (2% v/v) was introduced in the HC 2 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, six systems were considered for the AMF and AMF-T treatments without formaldehyde and four systems for the two treatments with formaldehyde. The 20 systems were incubated in a growth chamber under the same controlled conditions as above.

At harvest (i.e. 72 h 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 media of the RC and HC containing the fungal biomass were dissolved in citrate buffer (Doner & Bécard, 1991) 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 3 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).

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 into two groups of five 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 Experiment 1, respectively). Before harvest, hyphal interactions between *Glomus* sp. and *T. harzianum* were assessed through direct observations under a bright-field light microscope (Olympus BH-2, Olympus Optical GmbH) at × 50 magnification, i.e. without disturbing the three-dimensional configuration of the mycelium (De Jaeger *et al.*, 2010). 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 & Hayman (1970). Colonization was estimated under a bright-field light microscope at × 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 (Doner & Bécard, 1991) of the RC and HC. The hyphae were stained for ALP and SDH detection following the protocols of Olsson *et al.* (2002) and Saito (1995), respectively. Mycelium samples stained for ALP or SDH activity were mounted on a microscope slide with lactoglycerol. Granular precipitation of the samples was assessed using a bright-field light microscope (Olympus BH-2, Olympus Optical GmbH) at × 50 magnification. According to the magnified intersects method (McGonigle *et al.*, 1990), intersections of hyphae were classified as active or nonactive according to the visualization of granular precipitation. For each sample, at least 150 hyphal intersections were observed.

Statistical analysis

Data analysis was performed using the statistical software STATISTICA. 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 ($P \leq 0.05$).

Results

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

Phosphorus activities and specific activities measured in plant, fungal biomass and medium are reported in Table 1. At the end of the experiment, 12.0 ± 2.7% and 7.3 ± 2.2% of the ^{33}P initially supplied in the HC remained in the medium in the AMF and AMF-T treatments, respectively. This difference was significant between treatments. The ^{33}P activity measured in the AM fungal biomass in the HC was

Table 1. Phosphorus activities [count per minute (c.p.m.)] and specific activities measured on the shoot and roots of *Solanum tuberosum*, fungal biomass (i.e. hyphae from the AM fungus in the AMF treatment and from both fungi in the AMF-T treatment) in the RC, medium in the RC, fungal biomass (i.e. hyphae from the AM fungus in the AMF-T and AMF treatments) in the HC and medium in the HC

	AMF	AMF-T
Activity measured (c.p.m.)		
Shoot	37 483 ± 10 258b	11 505 ± 3131a
Roots	92 406 ± 10 464a	89 062 ± 22 632a
Fungal biomass (RC)	47 077 ± 4345a	39 866 ± 16 236a
Fungal biomass (HC)	309 345 ± 43 016a	330 104 ± 61 133a
Medium (RC)	21 248 ± 3437a	20 018 ± 4898a
Medium (HC)	119 822 ± 11 985b	72 787 ± 16 199a
Specific activity (c.p.m. mg^{-1})		
Shoot	237 ± 68a	70 ± 16b
Roots	3261 ± 592a	2643 ± 705a
Specific activity (c.p.m. cm^{-1})		
Fungal biomass (RC)	12 ± 1a	10 ± 4a
Fungal biomass (HC)	202 ± 36a	236 ± 46a

For each activity, values in the same line followed by identical letters do not differ significantly at $P \leq 0.05$ (Tukey HSD test). Data represent the means of six replicates (mean ± SE).

not significantly different and represented 59.8 ± 5.9% and 65.1 ± 9.1% of the ^{33}P taken up by the AM hyphae in the HC of the AMF and AMF-T treatments, respectively. 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 9.3 ± 8.5% and 8.3 ± 3.4% of ^{33}P taken up by the AM hyphae in the HC, respectively, and was not significantly different. No significant differences in the ^{33}P-specific activity of the fungal biomass of the HC and the RC were observed between the treatments (Table 1). Similarly, the ^{33}P content of the roots did not differ among the two treatments and represented 18.7 ± 2.8% and 19.5 ± 6.0% of ^{33}P taken up by the AM hyphae in the HC of the AMF and AMF-T treatments, respectively. No significant difference in the ^{33}P-specific activity in the roots was observed between the treatments (Table 1). In contrast, the ^{33}P content measured in the shoots was significantly higher in the AMF treatment as compared with the AMF-T treatment and represented 7.7 ± 2.3% and 2.5 ± 0.7% of ^{33}P taken up by the AM hyphae in the HC, respectively. A significant difference in the ^{33}P-specific activity in the shoot was identically observed between the treatments (Table 1). The ^{33}P released into the RC medium did not differ among the treatments and represented 4.2 ± 0.7% and 4.4 ± 1.2% of ^{33}P taken up by the AM hyphae in the HC for the AMF and AMF-T treatment, respectively.

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.

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 ERM) of the AM fungal mycelium by *T. harzianum* were observed in the RC (data not shown). *Trichoderma harzianum* developed profusely on the surface of the RC already 4 days after the start of the experiment and densely covered the entire surface within 7 days.

The AM development in the RC and in the HC did not differ significantly in the presence or in the absence of *T. harzianum*. Four days after *T. harzianum* inoculation (at ^{33}P labelling time), the AM mycelium covered the entire surface of the HC (i.e. 25.2 cm^2) in the presence as well as in the absence of the saprotrophic fungi. In the RC, the number of spores was 4743 ± 164 and 4632 ± 268 and the length of hyphae was 3316 ± 128 and 3282 ± 207 cm in the absence or in the presence of *T. harzianum*, respectively. In the HC, the number of spores was 125 ± 27 and 90 ± 20 and the length of hyphae was 1485 ± 96 and 1407 ± 93 cm in the absence or in the 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 ± 195 and 5119 ± 213 and the length of hyphae was 3831 ± 104 and 3703 ± 199 cm in the absence or in the presence of *T. harzianum*, respectively. In the HC, the number of spores was 142 ± 29 and 93 ± 19 and the length of hyphae was 1598 ± 91 and 1439 ± 93 cm in the absence or in the presence of *T. harzianum*, respectively.

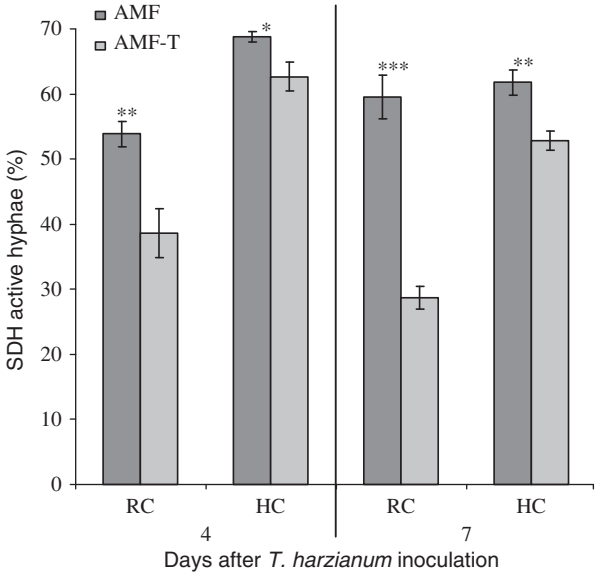

Fig. 2. Proportion of succinate dehydrogenase (SDH) activity determined in the extraradical hyphae of *Glomus* sp. MUCL 41833 in the absence (AMF treatment) or in the presence (AMF-T treatment) of *Trichoderma harzianum* MUCL 29707 (mean ± SE, $n = 5$) 4 and 7 days after inoculation of the saprotrophic fungus. Analyses were conducted in the RC and HC. Asterisks indicate a significant difference (*, ** and *** correspond to $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively; Tukey's honest significant difference test) between the treatments.

The presence of *T. harzianum* in the RC decreased the mycorrhizal viability (SDH staining) significantly in the RC and in the HC (Fig. 2). The proportion of AM viable hyphae in the RC was reduced by 28.5% and 51.7% in the AMF-T treatment as compared with 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 with the AMF treatment 4 and 7 days after *T. harzianum* inoculation, respectively.

Similarly, a significant difference was found in the mycorrhizal metabolic activity (ALP staining) between the two treatments (Fig. 3). 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

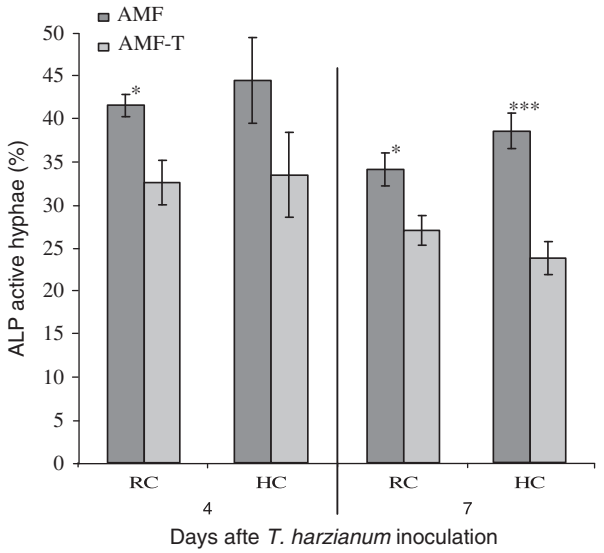

Fig. 3. Proportion of alkaline phosphatase (ALP) activity determined in the extraradical hyphae of *Glomus* sp. MUCL 41833 in the absence (AMF treatment) or in the presence (AMF-T treatment) of *Trichoderma harzianum* MUCL 29707 (mean ± SE, $n = 5$) 4 and 7 days after inoculation of the saprotrophic fungus. Analyses were conducted in the RC and HC. Asterisks indicate a significant difference (* and *** correspond to $P \leq 0.05$ and $P \leq 0.001$, respectively; Tukey's honest significant difference test) between the treatments.

Table 2. Root colonization of *Solanum 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

	4		7	
Time (days)	AMF	AMF-T	AMF	AMF-T
Total colonization (%)	56.5 ± 4.1a	57.3 ± 3.4a	62.6 ± 6.2a	57.1 ± 2.5a
Arbuscular colonization (%)	21.3 ± 1.6a	24.0 ± 2.0a	28.9 ± 4.9a	25.4 ± 1.1a
Vesicular colonization (%)	5.8 ± 1.0a	6.6 ± 1.9a	10.1 ± 1.7a	9.0 ± 0.7a

For each time (i.e. 4 or 7 days after *Trichoderma harzianum* inoculation), the 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 ± SE).

compared with 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 with the AMF treatment.

No differences in the total potato root colonization, arbuscular and vesicular colonization were observed between the treatments during the experimental period (Table 2).

Discussion

The interactions between mycorrhizal fungi and other soil organisms are complex. If beneficial interactions have been frequently mentioned (Bonfante & Anca, 2009), several authors have also reported antagonistic interactions with bacteria, fungi and microarthropods that may affect the functioning of the AM symbioses (Purin & Rillig, 2008; Bonkowski *et al.*, 2009; Miransari, 2011). Recently, De Jaeger *et al.* (2010) 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 bidirectional transport of C and P between both partners of the AM symbiosis. Under 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 the presence of the saprotroph. More P was taken up by the AM fungus in the presence of *T. harzianum*, while less P accumulated in the shoots of the potato plants. This observation was paralleled by a reduction of the viability (measured via SDH staining) and metabolic activity (measured via ALP staining) of the AM extraradical hyphae in the presence of *T. harzianum*.

Radiotracer studies have demonstrated that phosphate uptake by the ERM 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 & 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 polyphosphate chains (Ezawa *et al.*, 2001, 2004). The polyphosphates 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 the presence of *T. harzianum* in the RC, a consequence of the higher uptake by the AM ERM in the 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 with an overall lower transport of P by the AM fungus to the host plant seems contradictory and excludes the uptake as being the cause for this lower transport of P. Purin & Rillig (2008) hypothesized that mycoparasitized AM fungi (i.e. by *T. harzianum*) may compensate metabolite 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 could thus explain our observation. For instance, it could be suggested

that in response to AM mycelium parasitism, the expression or the activity of phosphate transporters could be increased. The regulation of P transporter gene expression coding for a protein belonging to the Pi/H^+ cotransporters 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 RC may regulate P uptake by the extraradical hyphae in the HC, suggesting communication between the different parts of the mycelium subject to the differing environmental conditions (Hodge *et al.*, 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.

Because of parasitism of the extra- and intraradical mycelium (De Jaeger *et al.*, 2010), 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 in AM viability and metabolic activity in the 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.*, 2010), indicating that *T. harzianum* was feeding on the AM fungus as reported by Benhamou & 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.* (2010) 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 & 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 ERM of AM fungi, mycophage animals such as Collembola affected the functionality of AM symbiosis (Hattingh *et al.*, 1973; Warnock *et al.*, 1982; Fitter & Sanders, 1992; Fitter & 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 ERM of an AM fungus. Similarly, Larsen & Jakobsen (1996) reported a decrease of 12% in the average hyphal ^{32}P transported from a root-free soil compartment to a plant compartment and a decrease of 30% of the average AM hyphal length in the presence of Collembola. However, in their experiment, the shoot yield was unaffected by the grazing, suggesting that Collembola had little effect on the functioning of AM fungi. 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 the 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 ERM could partly explain the decrease in AM viability (SDH staining) and activity (ALP staining) noted in the 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 & 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 because the AM symbiosis was established before the introduction of the saprotrophic fungus as reported earlier (McAllister *et al.*, 1994a; Vázquez *et al.*, 2000). Under similar experimental conditions, De Jaeger *et al.* (2010) 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 & 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. A 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, a 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 the root surface, interrupting the hyphal continuity with the intraradical mycelium, relatively low levels of hyphal predation could have drastic effects on mycorrhizal function. As suggested by Fitter & 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). It is obvious that these results should be confirmed under soil conditions to reflect the complexity of microbial interactions. Indeed, if our data confirm the earlier study conducted by Rousseau *et al.* (1996) under *in vitro* culture conditions, they seem to be in contradiction with the report of Green *et al.* (1999). These authors noted that growth and P uptake by the ERM of an AM fungus was not affected by *T. harzianum*, while the latter seemed to be impacted by the obligate symbiont. Nutritional and microbial environmental conditions as well as the strains of *Trichoderma* sp. and AM fungi used may explain the dissimilar results obtained.

In this study, we reported for the first time the impact of *T. harzianum* on the transport (i.e. uptake, translocation and transfer) of P by an AM fungus under rigorous *in vitro* culture conditions. 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 may also affect the plant performance indirectly by decreasing the source–sink relationship between the AM fungi and its host plant. Further studies should be conducted to ascertain these results under soil conditions.

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