Fenpropimorph and fenhexamid impact phosphorus translocation by arbuscular mycorrhizal fungi

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Abstract

Fenpropimorph and fenhexamid are sterol biosynthesis inhibitor (SBI) molecules widely used to control diseases in agriculture. Both molecules, at increasing concentrations, have been shown to impact on the non-target arbuscular mycorrhizal (AM) fungi. Root colonization, spore production and mycelium architecture, including the branched absorbing structures which are thought to be involved in phosphorus (P) uptake, were affected. In the present study, we investigated the capacity of *Glomus* sp. MUCL 43204 to take up, transfer and translocate labelled P to *Medicago truncatula* in the presence of these SBI molecules. We used a strict in vitro cultivation system associating an autotrophic plant of *M. truncatula* with the AM fungus. In addition, the effects of both SBI molecules on the proportion of hyphae with alkaline phosphatases (ALP), succinate dehydrogenase (SDH) activity and on the expression of the mycorrhiza-specific plant phosphate transporter MtPT4 gene were examined. We demonstrated that the two SBI molecules impacted the AM fungus. This was particularly evidenced for fenpropimorph. A decrease in P transport and ALP and SDH activities associated with the extraradical mycelium and *MtPT4* expression level was noted. These three factors were closely related to the development of the AM fungus, suggesting a direct impact not only on the AM fungal growth but also on the physiology and metabolic activities of the AM fungus. These results further emphasized the interest on the autotrophic in vitro culture system as an alternative to pot experiments to investigate the mechanisms behind the impact of disease control molecules on the non-target AM fungal symbionts.

Keywords

Alkaline phosphatasesArbuscular mycorrhizal fungiArbuscular mycorrhizal-plant (AM-P) in vitro culture systemsFenhexamidFenpropimorph*MtPT4* geneRoot organ cultureSterol biosynthesis inhibitorSuccinate dehydrogenase

Introduction

Arbuscular mycorrhizal (AM) fungi are ubiquitous soil microorganisms that form a symbiotic association with the roots of the majority of plant species (Smith and Read 2008). Through the extended development of their mycelium in soil, they facilitate the assimilation of less mobile nutrients, such as phosphorus (P), in exchange of which they receive carbohydrates from the plants (Parniske 2008). Their impact on plant growth, resistance to biotic and abiotic stresses and ecosystem functioning has been widely documented (Van der Heijden et al. 2006). Hence, they are nowadays considered as the most important plant symbionts, with nitrogen-fixing bacteria that "help feed the world" (Marx 2004).

The functioning of AM fungi may be impaired by cultural practices such as fungicides application (Sukarno et al. 2006). Among fungicides, the sterol biosynthesis inhibitors (SBI) family is one of the most used in agriculture. Fenpropimorph is active on, e.g., rusts and powdery mildew of cereals, while fenhexamid is used against, e.g., *Botrytis cinerea* of grape and *Monilia* spp. of stone fruits. Since the utilization of SBI fungicides is targeted at the control of fungal pathogens, most studies have been conducted without paying attention to AM fungi. Recent works using root organ cultures have described how these SBI fungicides affect the AM symbiosis (Zocco et al. 2008; Campagnac et al. 2008, 2009, 2010; Oger et al. 2009). Fenpropimorph presented a high toxicity with drastic sterol modifications in the host roots which were mirrored by a drastic reduction of root growth and root colonization. By contrast, the application of fenhexamid did not modify the sterol profiles and had a very limited impact on mycorrhization (Campagnac et al. 2008). The increased concentrations of fenpropimorph and fenhexamid had an impact on the extraradical fungal development: hyphal growth, spore production and mycelium architecture were strongly affected (Zocco et al. 2008), possibly due to a direct impact on the fungal sterol metabolism (Campagnac et al. 2009). In particular, the branched absorbing structures (BAS), thought to be involved in P uptake (Bago 2000), were affected both quantitatively and in the level of branching (Zocco et al. 2008). In addition, SBI fungicides have a physiological impact on membranes (Martin and Konopka 2004) which can lead to a potential interference with transport activities occurring across membranes. These data therefore suggest that SBI molecules can impact on the capacity of the AM fungus to take up, translocate and transfer P from the environment to its host plant.

The transfer of phosphate (Pi) from the AM fungus to the plant involves different steps and enzymes (Javot et al. 2007a). In extraradical hyphae, Pi enters the cytoplasm via high-affinity $Pi:H^+$ symporters (Harrison and Van Buuren 1995; Javot et al. 2007a) and is rapidly converted into polyphosphate (poly-P) (Ezawa et al. 2003). Following translocation from the extraradical mycelium to the intraradical mycelium, poly-P is thought to be hydrolysed by phosphatases. Hydrolysis of poly-P at sites of P utilization or transfer to the plant probably occurs through the combined activities of exopolyphosphatases, endopolyphosphatases and non-specific acid phosphatases (ACP) (Smith and Read 2008). Alkaline phosphatases (ALP) and ACP activities have also been detected in extra- and intraradical hyphae (Smith and Gianinazzi-Pearson 1990; van Aarle et al. 2001; Ezawa et al. 2001; Aono et al. 2004). The final steps of the process require the release of Pi into the peri-arbuscular apoplastic compartment and the transfer to plant cells via membrane transporters.

At present, two classes of plant Pi transporters belonging to the Pht1 family have been described to be involved in the Pi transport in the AM symbiosis: the mycorrhiza-specific and the mycorrhiza-up-regulated Pi transporters (Javot et al. 2007a). A member of subfamily I, *MtPT4* of *Medicago truncatula*, is strictly expressed in response to AM root colonization, in particular in arbuscule-containing cells (Harrison et al. 2002). *MtPT4* activity was shown to be essential for the acquisition

of Pi delivered by the AM fungus and for the correct development of the fungus inside the roots (Javot et al. 2007b).

Recently, Dupré de Boulois et al. (2006) developed a strict in vitro cultivation system associating an autotrophic plant with an AM fungus. This system appeared adequate to investigate the transport of radiocaesium as well as P from a labelled compartment to the host plant, via the mycorrhizal network, and opened the door for studies involving the impact of abiotic constraints such as fungicide molecules on P transport.

In the present study, we investigated the capacity of an AM fungus (i.e. *Glomus* sp. MUCL 43204) to take up, transfer and translocate labelled P to *M. truncatula* in the presence of fenpropimorph and fenhexamid. In addition, the effects of both SBI molecules on the activity of the fungal ALP and succinate dehydrogenase (SDH) and on the expression of the plant *MtPT4* gene were examined.

Materials and methods

Biological material

The AM fungus used was MUCL 43204, formerly identified as *Glomus intraradices* and presently reclassified in a clade that contains the recently described species *Glomus irregulare* Błaszk., Wubet, Renker, and Buscot (Stockinger et al. 2009). The relationships among fungi in this group are not yet clear, and consequently we continue to use the MUCL designation rather than to use a Linnaean binomial that may turn out to be in need of revision in the future. The AM fungus strain MUCL 43204 was purchased from GINCO (http://www.mbla.ucl.ac.be/ginco-bel) and grown in association with Ri T-DNA transformed carrot (*Daucus carota* L.). The strain was supplied in Petri plates (90 mm in diameter) on the modified Strullu–Romand (MSR) medium (Declerck et al. 1998) solidified with 3 g l^{-1} Gel Gro™ (ICN, Biomedicals, Irvine, CA, USA). The Petri plates were incubated in an inverted position at 27°C in a growth chamber under dark conditions. Several thousands of spores were produced within 4 months.

Seeds of *M. truncatula* L., cv. Jemalong A17, were surface-sterilized by immersion in sodium hypochlorite (8% active chloride) for 10 min and rinsed in deionized sterile (121°C for 15 min) water (Dupré de Boulois et al. 2006). Seeds were then placed in Petri plates on the surface of MSR medium (10 ml) lacking sucrose and vitamins (termed MSR1) and solidified with 4 g l^{-1} Gel Gro™. Germination was conducted in a growth chamber set at 20°C in the dark for 10 days.

SBI molecules

Fenpropimorph and fenhexamid (technical grade) were supplied by BASF and Bayer CropScience, respectively. For each experiment, the two SBI molecules were dissolved in a solution of acetone (0.5 ml l^{-1} MSR1) and subsequently added to sterilized (121°C for 15 min) MSR1 molten medium (80°C), solidified with 4 g l^{-1} Gel Gro™, in order to obtain, in final concentration, 0.2 and 2 mg l^{-1} of both SBI molecules (Zocco et al. 2008). A control treatment (MSR1 medium supplemented with acetone but without SBI molecules) was also included in the experiment.

Experimental design

Ten-day old *M. truncatula* plantlets, with shoots of approximately 50 mm in length and primary roots of approximately 70 mm in length having two to five secondary roots, were transferred to the arbuscular mycorrhizal-plant (AM-P) in vitro culture systems (Dupré de Boulois et al. 2006).

Briefly, the system consisted of a plastic falcon tube (referred as the shoot compartment, SC) cut at the bottom and glued to an opening made on the lid of a bi-compartmental Petri plate. The Petri plate was divided into a root compartment (RC) and a hyphal compartment (HC). The shoot developed in the SC. The root system with or without AM fungi was restricted to the RC, whilst the AM fungal extraradical mycelium was allowed to cross the plastic barrier separating the RC from the HC to develop into the HC.

Shoots of *M. truncatula* were inserted in the opening between the SC and the RC with their roots placed in the RC on the surface of the MSR medium lacking sucrose and vitamins. Roots were subsequently associated with ±100 spores of the AM fungus following solubilisation of the gellan gel (Doner and Becard 1991). The Petri plates were then sealed with parafilm (Pechiney, Menasha, WI, USA) and wrapped in dark plastic bags. The Petri plates were incubated in a controlled growth chamber set at 20°C with a 16-h photoperiod. Daylight lamps provided an average photosynthetic photon flux at the level of the SCs of 225 µmol m^{-2} s^{-1}.

Three weeks after association, the AM fungus had established numerous contact points with the roots and started to colonize the total surface of the RC. Five weeks after association, the HCs were filled with 25 ml of MSR medium, lacking sucrose and vitamins, without SBI molecule or supplemented with 0.2 mg and 2 mg l^{-1} of either/one of the two SBI molecules and solidified with 4 g l^{-1} Gel Gro™. Within a period of 3 days, the AM fungus crossed the partition wall separating the RC from the HC. Eight weeks after association (i.e. 3 weeks after the AM fungus developed in the HC), the AM-P in vitro culture systems were divided in three sub-groups for the study of (1) P transport, (2) enzyme histochemical staining and (3) *MtPT4* gene expression analysis.

Transport of phosphorus

One sub-group of plants was used to investigate the P transport. Transport of ^{33}P was studied in two independent experiments for fenpropimorph and fenhexamid. For the fenpropimorph treatment (at 0.2 and 2 mg l^{-1}) and the control (i.e. without SBI molecule), seven replicates were considered. For the fenhexamid treatment (at 0.2 and 2 mg l^{-1}) and for the control (i.e. without SBI molecule), six replicates were considered. In both experiments, a source of P (^{33}P) was added to the HCs. The source of ^{33}P was filter-sterilized (Acrodisc Syringe filters, PALL Corporation, Ann Arbor, MI, USA) and directly applied on the surface of the MSR1 medium. The final activity in the MSR1 medium at the start of the two experiments was 858 Bq and 1,395 Bq ml^{-1} of MSR1 medium for the fenpropimorph and fenhexamid, respectively. The difference in total activity at the start of the two experiments was related to the short half-life of ^{33}P (19 days). The source of ^{33}P was orthophosphate in diluted hydrochloric acid (<0.1 M) as supplied by PerkinElmer (Zaventem, Belgium). A formaldehyde control was also considered for each concentration of both SBI molecules. The formaldehyde control aimed to determine whether the translocation of ^{33}P from the HC to the RC was mediated by active processes. Formaldehyde (2% *v*/*v*) was introduced 2 days before the addition of ^{33}P in the HC (Dupré de Boulois et al. 2005).

The experiment was conducted for 72 h according to earlier experiments conducted with root organ cultures (Nielsen et al. 2002) showing a large amount of ^{32}P transported from RC to HC within less than 72 h. At the end of both experiments (i.e. 72 h after ^{33}P addition), the surface of mycelium covering the HC, the number of spores in the HC and the number of active hyphae (i.e. presenting bi-directional cytoplasmic flux) crossing the RC to the HC were estimated for each replicate. The surface of the extraradical mycelium covering the HC was estimated using a transparent plastic sheet placed on the bottom of the Petri plates. The surface area in the HC covered by the extraradical mycelium was traced on the sheet. The surface was then estimated as compared to the whole surface of the HC (Voets et al. 2009). Spore counting was performed following the method

of Declerck et al. (2001). The number of active hyphae (observed as bi-directional cytoplasmic flux) crossing the plastic barrier separating the RC from the HC was measured under a dissecting microscope (Olympus SZ40, Olympus Optical (Europa) Gmbh, Germany) at ×40 magnification. Shoots of *M. truncatula* were harvested by cutting the aerial part at the level of the medium in the RCs. The medium contained in the HCs and in the RCs was sub-sampled. Mycelium growing into the HCs in the presence of both concentrations of SBI molecules was harvested and separated from the MSR1 medium following solubilisation of the gel (Doner and Becard 1991). Roots were also separated from the MSR1 medium. Shoots fresh weight and root fresh weight of the plants were measured.

Root colonization by the AM fungus was estimated under a compound microscope (Olympus BH2, Olympus Optical (Europa) Gmbh, Germany) at ×50 to ×125 magnifications following the method of McGonigle et al. (1990). The roots were first cleared with 10% KOH and then stained with 0.1% Trypan blue overnight at 20°C. Root colonization was assessed by evaluating the total root colonization and the proportion of root length containing arbuscules (%A), vesicles (%V) and hyphae (%H) with an average number of intersections of 180.

The transport of ^{33}P was assessed by counting the activity in the MSR1 medium (on a sub-sample of 1 ml) of the HCs and RCs, in the AM fungal biomass of the HCs, in the roots of the RCs and in the shoots of the SCs. The plant and fungal samples were first digested in perchloric acid 70%/nitric acid 30% in solution 1:1 and clarified with hydrogen peroxide at 30% as described by Becker et al. (1992). An aliquot of 10 ml of liquid scintillation cocktail was added to the samples (Ultima Gold™, Packard BioScience, Groningen, The Netherlands). The samples were then counted for ^{33}P activity on a Packard TR2500 Liquid Scintillation Analyser (Packard Instruments, Meriden, CT, USA).

Enzyme histochemical staining

One sub-group of plants was used for histochemical staining. In each replicate, five samples of mycelium (from 9-mm diameter) were collected in the HCs with a cork borer. The samples were dissolved in citrate buffer and rinsed thoroughly to remove all traces of citrate buffer. The extraradical mycelium of the fenpropimorph and fenhexamid (at 0.2 and 2 mg l^{-1}) treatments and the control (i.e. without SBI molecule) treatment was stained for alkaline phosphatase and succinate dehydrogenase detection. The SDH staining method enabled us to evaluate the proportion of metabolically active extraradical hyphae.

For ALP activity measurement, a 0.1-M Tris–HCl buffer (pH 8.5) with 1.1 mg ml^{-1} α-naphthyl phosphate Na salt (Sigma) and 1.0 mg l^{-1} fast blue RR (Sigma) was used (Van Aarle et al. 2002). The samples were incubated for 90 min at RT in the dark in the staining solution, washed with deionised water, counterstained with 0.1% acid fuchsin in lactic acid and transferred to lactoglycerol for overnight de-staining.

For SDH activity measurement, stock solutions of 0.2 M Tris/HCl (pH 7.4), 5.0 mM $MgCl_2$ and 1.0 M sodium succinate were prepared (Smith and Dickson 1997). A working solution of 2.5 ml Tris–HCl, 1.0 ml $MgCl_2$, 2.5 ml sodium succinate, 4.0 ml H_2O and 10 mg NBT was freshly prepared (Smith and Dickson 1997). The samples were incubated overnight at RT in the dark in the staining solution, washed with deionised water, counterstained in 0.1% acid fuchsin in lactic acid for 15 min and then transferred to lactoglycerol for de-staining.

Mycelium samples stained for either ALP or SDH activity were mounted on microscope slides with lactoglycerol. The precipitation of substrate after enzymatic reaction was assessed with a

microscope (Olympus BH2) with a bright-field view at ×500 magnification. For each sample, an approximate of 180 hyphal intersections was observed. According to Van Aarle et al. (2002), the intersections of hyphae were classified as active or non-active, following substrate precipitation or not.

For fenhexamid (0.2 and 2 mg l^{-1}) and the control (i.e. without SBI molecule), five replicates were considered for ALP and SDH. With fenpropimorph, the mycelial growth was highly impaired and the number of replicates differed with concentration. For ALP and SDH staining, five and two replicates were considered at concentrations of 0.2 and 2 mg l^{-1} of fenpropimorph, respectively.

Gene expression analysis

One sub-group of plants was used for *MtPT4* gene expression analysis. RNA was extracted from 100 mg mycorrhizal roots using the TRIZOL Reagent (Sigma) and the Pure Link™ Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The RNA was precipitated with 6 M LiCl and re-suspended in 20 µl of sterile water. The RNA samples were routinely checked for DNA contamination by RT-PCR analyses conducted using the primers for the *M. truncatula* glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (forward: 5′-GCTAGCACTGGTGCTGATATT-3′ and reverse: 5′-TTCCTTCTCATTGACACCAAC-3′) and the One-Step RT-PCR kit (QIAGEN). Reactions were carried out in a final volume of 10 µl containing 2 µl of 5X buffer, 400 µM dNTPs, 0.8 µM of each primer, 0.4 µl of One-Step RT-PCR enzyme mix and 1 µl of total RNA. In the case of DNA contamination, samples were treated with RNase-free deoxyribonuclease I (Invitrogen) according to the manufacturer's instruction.

The first single-strand cDNA was primed by random hexamers using 100 ng of total RNA. RNA samples were denatured at 65°C for 5 min and then reverse-transcribed at 25°C for 10 min and 42°C for 50 min in a final volume of 20 µl containing 11 µl of total RNA, 10 µM random primers (Invitrogen), 0.5 mM dNTPs, 4 µl of 5X buffer, 2 µl 0.1 M DTT and 1 µl of SuperscriptII (Invitrogen).

To construct standard curves in real-time experiments, recombinant plasmids containing a portion of the gene of interest (MtPT4 and GAPDH) were obtained by cloning cDNA fragments amplified by conventional PCR. The conventional PCR reactions were carried out in a final volume of 20 µl containing 10 mM of Tris–HCl, pH 8.3, 50 mM of KCl, 1.1 mM of $MgCl_2$, 0.01% of gelatin, 200 µM of each dNTP, 1 µM of each primer (MtPT4 forward 5′-TCGCGCGCCATGTTTGTTGT-3′ and MtPT4 reverse 5′-CGCAAGAAGAATGTTAGCCC-3′; *GAPDH* primers as described above), 50–100 ng of cDNA and 1 U of Hotstart *Taq* DNA polymerase (Qiagen). A PCR programme was conducted in a Perkin Elmer GeneAmp 9700 thermal cycler according to these parameters: 95°C for 15 min (1 cycle), 94°C for 45 s, 50°C for 45 s, 72°C for 1 min (35 cycles) and 72°C for 7 min (1 cycle). The negative controls for all the PCR experiments consisted of reaction mixtures from which template DNA was omitted. The PCR products were separated on 1.2% of TAE 0.5X agarose gel and visualized by ethidium bromide staining. The PCR products were purified from agarose gels using the QIAEX II Gel Extraction Kit (QIAGEN) and directly cloned in the pGEM-T vector (PROMEGA, Madison, WI, USA). Plasmid DNA was extracted using the Qiagen Miniprep Kit and sequenced by GENELAB (ENEA, Rome, Italy).

Individual real-time reactions were assembled in a final volume of 20 µl with 0.15 mM of each oligonucleotide, 10 µl of 2X iQ™ SYBR Green Supermix (BIO-RAD, Hercules, CA, USA) plus an appropriate volume of each cDNA preparation. The following primers were used: *G. intraradices 28S RNA* forward (5′-CGCCGATGTTTCTGCTGTTG-3′) and reverse (5′-

GCGACGTCCGAAGTTGCTTT-3′), the phosphate transporter *MtPT4* forward (5′-TCGCGCGCCATGTTTGTTGT-3′) and reverse (5′-CGCAAGAAGAATGTTAGCCC-3′) and the *M. truncatula* GAPDH and *MtPT4* primers described above.

The PCR cycling programme (15 s at 95°C followed by 30 s at 63°C) included a heating step (3 min at 95°C) at the beginning of each run. Real-time RT-PCR was carried out with an ICycler apparatus (BIO-RAD). A melting curve (55–95°C with a heating rate of 0.5°C per 10 s and a continuous fluorescence measurement) was recorded at the end of each run to assess amplification product specificity (Ririe et al. 1997). The reactions were performed on at least three independent biological samples with three technical replicates (only Ct values with a standard deviation below 0.3 were considered). The comparative threshold cycle (Ct) method (Rasmussen 2001) was used to calculate the *MtPT4* relative gene expression level. The *GAPDH* of *M. truncatula* was used as a housekeeping gene to normalize the samples.

Statistical analysis

Data were subjected to an analysis of variance. The homogeneity of variance was tested (Levene). The Tukey HSD test ($P < 0.05$) was used to discriminate among the means. All the tests were performed using the SAS statistic software.

Results

Transport of phosphorus

Plant and fungal growth parameters

At the end of both experiments (i.e. 8 weeks after association), the *M. truncatula* plants were still actively growing. New leaves and white and fine roots were observed in each treatment whatever the concentration of the SBI molecule added in the HC. With the exception of the shoot fresh weight (SFW) of fenpropimorph 2 mg l^{-1} treatment, the root fresh weight (RFW) and SFW did not differ between the control treatments and both SBI molecules treatments (Table 1). The extraradical mycelium development in the HC was highly impacted in the fenpropimorph treatment. At both concentrations, the number of hyphae crossing the plastic barrier separating the RC from the HC, the surface covered by the mycelium, the mycelium weight and the number of spores were significantly decreased as compared to the control treatment (Table 1). The data recorded for these parameters were further significantly lower in the fenpropimorph treatment at a concentration of 2 mg l^{-1} as compared to the fenpropimorph treatment at a concentration of 0.2 mg l^{-1} (Table 1). Root colonization (i.e. %total root colonization, %V, %A and %H) was significantly decreased at 0.2 mg l^{-1} fenpropimorph as compared to the control, while no difference was observed (with the exception of %V) with the fenpropimorph treatment at a concentration of 2 mg l^{-1} for % total root colonization, %A and %H (Table 1). The % total root colonization and %V did not differ between the two fenpropimorph treatments, while % A and %H was significantly decreased at the lowest concentration (i.e. 0.2 mg l^{-1}) as compared to the highest concentration (i.e. 2 mg l^{-1}) (Table 1).

Table 1 Plant and AM fungal growth variables measured as shoots fresh weight (SFW), roots fresh weights (RFW), total root colonization, proportion of root length with vesicles (%V), arbuscules (%A), hyphae (%H), number of spores, weights of extraradical mycelium, surface covered by the mycelium and number of active hyphae crossing the plastic barrier separating the root compartment from the hyphal compartment

		Fenpropimorph			**Fenhexamid**		
Compartment	**Parameter**	**Control (i.e. 0 mg l^{-1})**	**0.2 mg l^{-1}**	**2 mg l^{-1}**	**Control (i.e. 0 mg l^{-1})**	**0.2 mg l^{-1}**	**2 mg l^{-1}**
SC	SFW (mg)	514 ± 59[a]a	462 ± 84a	437 ± 43b	537 ± 22a	569 ± 47a	581 ± 37a
RC	RFW (mg)	606 ± 103a	551 ± 107a	595 ± 129a	471 ± 37a	495 ± 37a	484 ± 16a
	Total root colonization	32.11 ± 1.24a	16.19 ± 2.26b	26.7 ± 8.5ab	23.25 ± 2.96a	27.08 ± 5.08a	26.85 ± 4.85a
	%V	19.6 ± 1.31a	13.8 ± 0.38b	12.4 ± 3.73b	11 ± 3.14a	15.71 ± 4.21a	13.71 ± 4.11a
	%A	10.2 ± 0.084a	5.4 ± 0.98b	9.4 ± 3.78a	5.5 ± 1.46ab	4.09 ± 1.45b	7.42 ± 1.38a
	%H	18.2 ± 3.77a	10.2 ± 1.37b	18 ± 5.11a	7.37 ± 2.39a	15.71 ± 1.99b	8.85 ± 2.21a
HC	Number of spores	2,761 ± 532a	1,000 ± 336b	44 ± 39c	3,514 ± 751ab	4,195 ± 633a	2,896 ± 493b
	Mycelium weight (mg)	305 ± 43a	138 ± 34b	47 ± 19c	327 ± 21a	341 ± 12a	352 ± 12a
	Surface covered by the mycelium (%)	76.25 ± 6.50a	34.87 ± 9.53b	14.12 ± 3.98c	91 ± 4a	92.5 ± 3.22a	96 ± 2,44a
RC and HC	Number of active crossing hyphae	63 ± 14a	29 ± 13b	3 ± 1c	115 ± 21a	88 ± 16ab	59 ± 16b

Seven replicates were considered for the fenpropimorph experiment and six for the fenhexamid experiment. For each SBI molecule, values in the same line followed by identical letters do not differ significantly (Tukey test, $P < 0.05$)

[a]Values represent means ± standard error

At the lowest concentration of fenhexamid (i.e. 0.2 mg l^{-1}), no differences were observed with the control treatment for the number of hyphae crossing the plastic barrier separating the RC from the HC, the surface covered by the mycelium, the mycelium weight and the number of spores as compared to the control (Table 1). At 2 mg l^{-1} of fenhexamid, a significant difference with the control treatment was observed for the number of hyphae crossing the plastic barrier separating the RC from the HC (Table 1), while no significant differences were observed for the other parameters. With the exception of the number of spores produced (significantly higher at concentration

0.2 mg l^{-1} as compared to 2 mg l^{-1}), no significant differences were observed between the two fenhexamid treatments (Table 1). The % total root colonization and %V did not differ between the control treatment and the fenhexamid treatments (at 0.2 and 2 mg l^{-1}) (Table 1). The % A and %H did not differ between the control and the fenhexamid treatment at a concentration of 2 mg l^{-1}, while at 0.2 mg l^{-1} of fenhexamid both parameters were significantly decreased as compared to the control (Table 1).

Phosphorus transport

The presence of fenpropimorph in the HC significantly reduced the capacity of the AM fungus to transfer the ^{33}P added to the HC. In the control treatment (i.e. without SBI molecule), the uptake by the extraradical mycelium developing in the HC amounted for ~87% of the initial supply of ^{33}P in the HC. This percentage significantly decreased to ~77% and ~44% of the initial ^{33}P supplied in the HC in the presence of 0.2 and 2 mg l^{-1} fenpropimorph, respectively (Table 2).

Table 2 Phosphorus activities (Bq) and specific activities measured on shoots in the shoot compartment (*SC*), roots and solid MSR medium lacking sucrose and vitamins in the root compartment (RC), AM fungal biomass and solid MSR medium lacking sucrose and vitamins in the hyphal compartment (HC)

Activity (Bq) measured in the compartment		**Fenpropimorph**			**Fenhexamid**		
		Control (i.e. 0 mg l^{-1})	**0.2 mg l^{-1}**	**2 mg l^{-1}**	**Control (i.e. 0 mg l^{-1})**	**0.2 mg l^{-1}**	**2 mg l^{-1}**
SC	Shoots	888 (4.14 ± 1.34) [a]a	710 (3.31 ± 1.05) a	114 (0.53 ± 0.28)b	290 (1.02 ± 0.33) a	217 (0.76 ± 0.26) a	281 (0.98 ± 0.31)a
RC	Roots	7,679 (35.79 ± 6.92)a	8,093 (37.72 ± 7.98)a	4,325 (20.15 ± 8.46) b	3,455 (12.14 ± 2.02)a	2,513 (8.83 ± 1.43) a	3,471 (12.19 ± 1.94) a
HC	Solid medium with fungal biomass	337 (1.57 ± 0.33) a	255 (1.18 ± 0.48) a	77 (0.36 ± 0.12)b	2,476 (8.70 ± 4.49) a	1,822 (6.40 ± 2.36) a	1,550 (5.44 ± 0.97)a
	Fungal biomass (total activity and per mg of myceliu m)	7,631 (35.57 ± 2.81)a	7,509 (35 ± 10.95)a	3,821 (17.81 ± 5.12) b	13,310 (46.76 ± 5.85)a	10,472 (36.79 ± 9.84)a	13,414 (47.13 ± 11.40)a
		25.02 ± 1.98[b] a	54.41 ± 17.0 2b	81.31 ± 23.4b	40.70 ± 8.24 a	30.71 ± 4.48 a	38.10 ± 7.72a
	Solid medium	2,781 (12.96 ± 2.45)a	4,910 (22.89 ± 6.95)b	12,114 (56.46 ± 10.51)c	8,928 (31.37 ± 4.97)a	9,926 (34.87 ± 5.59)a	8,972 (31.52 ± 4.37) a

[a]Activity in Bq (percentage ± standard error). Values in parenthesis correspond to the percentages ± the standard errors (SE) of the ^{33}P initially supplied in the HCs of fenpropimorph- and fenhexamid-

treated AMP in vitro culture systems and measured in shoots, roots, extraradical mycelium and MSR (HCs and RCs)

[b]Activity in Bq ± standard error, estimated per milligramme of fungal mycelium. Seven replicates were considered for the fenpropimorph experiment and six for the fenhexamid experiment. For each SBI molecule, values in the same line followed by identical letters do not differ significantly (Tukey test, $P < 0.05$)

The reduced uptake P may be largely due to the decrease in fungal biomass observed in the fenpropimorph treatment (Table 1). For this reason, phosphorus activity per fungal biomass (Bq/µg mycelium) was also calculated (Table 2): from these values, it seems that fenpropimorph increases the capacity of the fungus to take up P. We might conclude that the fungus can rapidly take up P also in the presence of fenpropimorph. However, despite of this uptake capacity, P translocation seems to be affected by this fungicide.

An average of ~35%, ~37% and ~20% of the initial ^{33}P supplied in the HC was translocated within the mycorrhizal roots developing in the RC for the control or treatments supplemented with 0.2 and 2 mg l^{-1} fenpropimorph, respectively. The transfer of ^{33}P to the shoots represented ~4%, ~3% and ~0.5% of the initial supply of ^{33}P in the HC for the control or treatments supplemented with 0.2 and 2 mg l^{-1} fenpropimorph, respectively.

At the highest concentration of fenpropimorph, ~56% of the ^{33}P added to the HC remained in this compartment, while this percentage decreased to ~23% and ~13% at the lowest concentration of fenpropimorph and for the control, respectively. The metabolic activity of the AM fungus, evaluated by microscopic observation of cytoplasmic/protoplasmic movement, was immediately stopped after the addition of formaldehyde to the HC (i.e. the formaldehyde control). In this control treatment, only traces of ^{33}P were measured (less than 0.01%) in the mycorrhizal roots of the RC, in the presence as well as absence of fenpropimorph (data not shown).

In contrast to fenpropimorph, increasing the concentration of fenhexamid in the HC did not impact on the capacity of the AM fungus to take up, translocate and transfer ^{33}P to the HC. No significant differences were observed between the treatments (Table 2). In the control treatment, ~69% of the initial ^{33}P supplied to the HC was taken up by the AM fungus. This percentage was ~65% and ~68% in the presence of 0.2 and 2 mg l^{-1} of fenhexamid, respectively. An average of ~12%, ~9% and ~12% (i.e. control, 0.2 and 2 mg l^{-1} fenhexamid, respectively) of the total ^{33}P supplied to the HC was translocated within the roots developing in the RC. An activity corresponding to ~1%, ~0.76% and ~1% of the initial supply of ^{33}P in the HC was also measured, 72 h after labelling, in the shoots of the mycorrhizal plants for the control or treatments supplemented with 0.2 and 2 mg l^{-1} fenhexamid, respectively. Almost no activity (less then 0.01%) was measured in the formaldehyde treatment (data not shown).

Alkaline phosphatase and succinate dehydrogenase activity

Increasing the concentration of fenpropimorph highly impacted on the activity of ALP (Fig. 1a). In the control treatment, the proportion of extraradical hyphae exhibiting ALP activity was 87.5% and significantly decreased to 36.5% and 18.1% at 0.2 and 2 mg l^{-1} of fenpropimorph, respectively. Identically, the proportion of hyphae exhibiting ALP activity in the control treatment of the fenhexamid experiment was significantly higher, i.e. 95.6%, than in the fenhexamid treatments (Fig. 1b), while no significant differences were observed between 0.2 and 2 mg l^{-1} of fenhexamid (Fig. 1b). At both concentrations, the ALP activity was 90.7%.

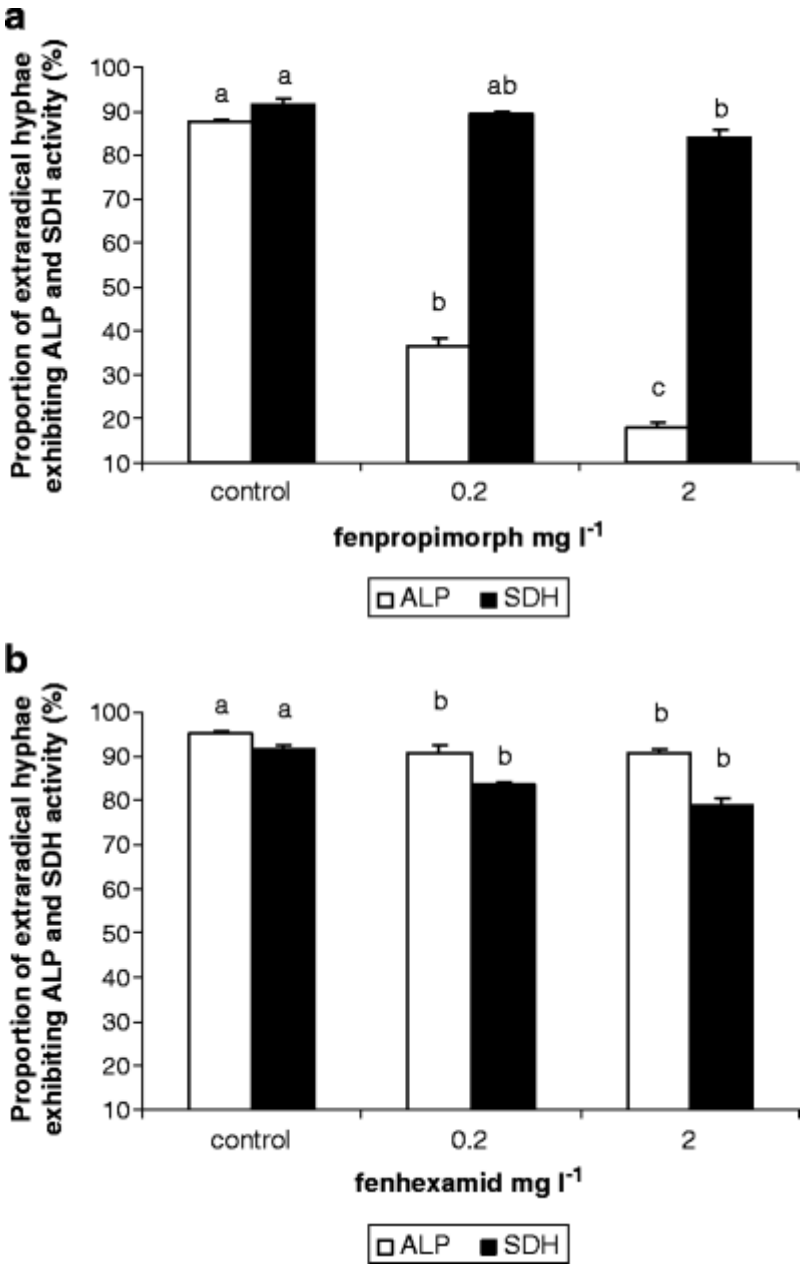

Fig. 1 Effects of increasing concentrations of fenpropimorph (**a**) and fenhexamid (**b**) on the proportion of extraradical hyphae exhibiting alkaline phosphatase (*ALP*) and succinate dehydrogenase (*SDH*) activity. For both enzymes, *histograms* with identical letters do not differ significantly ($P < 0.05$). For fenhexamid, five replicates were considered for ALP and SDH. With fenpropimorph, the mycelial growth was highly impaired and the number of replicates differed with concentration. For ALP and SDH staining, five and two replicates were considered at concentrations of 0.2 and 2 mg l^{-1} of fenpropimorph, respectively. For ALP and SDH, *bars* with the same letters are not significantly different (Tukey test, $P < 0.05$)

The proportion of hyphae exhibiting SDH activity did not significantly differ between the control (i.e. 91.6%) and the fenpropimorph treatment at a concentration of 0.2 mg l^{-1} (i.e. 89.2%), while this proportion of hyphae exhibiting activity was significantly higher as compared to the concentration of 2 mg l^{-1} of fenpropimorph (i.e. 84%) (Fig. 1a). The proportion of hyphae exhibiting SDH activity in the control treatment was significantly higher (i.e. 91.5%) compared to both fenhexamid treatments, while no significant differences were observed between 0.2 and 2 mg l^{-1} of fenhexamid (83.5% and 78.9%, respectively; Fig. 1b).

MtPT4 gene expression profiles

RNAs were extracted from a pool of root pieces representing almost the whole root system of single plants. Quantitative real-time RT-PCR was used to monitor *MtPT4* transcript levels and to evaluate the presence of the AM fungus on the basis of its ribosomal 28S rRNA. This was done considering that Isayenkov et al. (2004) found in *M. truncatula* colonized by *G. intraradices* a strong correlation between (1) fungal root colonization and amount of fungal rRNA and (2) *MtPT4* transcript level and presence of arbuscules.

Based on fungal rRNA abundance, the degree of root colonization in the different analysed plants was rather variable in all treatments. However, a good correlation was found between fungal rRNA abundance and *MtPT4* transcript levels in the control and the 0.2 mg l^{-1} fenhexamid samples. For several plants of the 2 mg l^{-1} fenhexamid treatment, colonization levels similar to those observed in the control samples were accompanied by lower *MtPT4* transcript levels (Fig. 2a). Thus, at the highest fenhexamid concentration, no linear relationship was observed between the colonization degree and the *MtPT4* expression levels. By contrast, in 0.2 and 2 mg l^{-1} fenpropimorph treatments, a strong decrease in the *MtPT4* mRNA levels was observed compared to the control treatment. This low *MtPT4* transcript level correlated with the level of the fungal 28S rRNA that was almost not detectable at both fungicide concentrations (Fig. 2b).

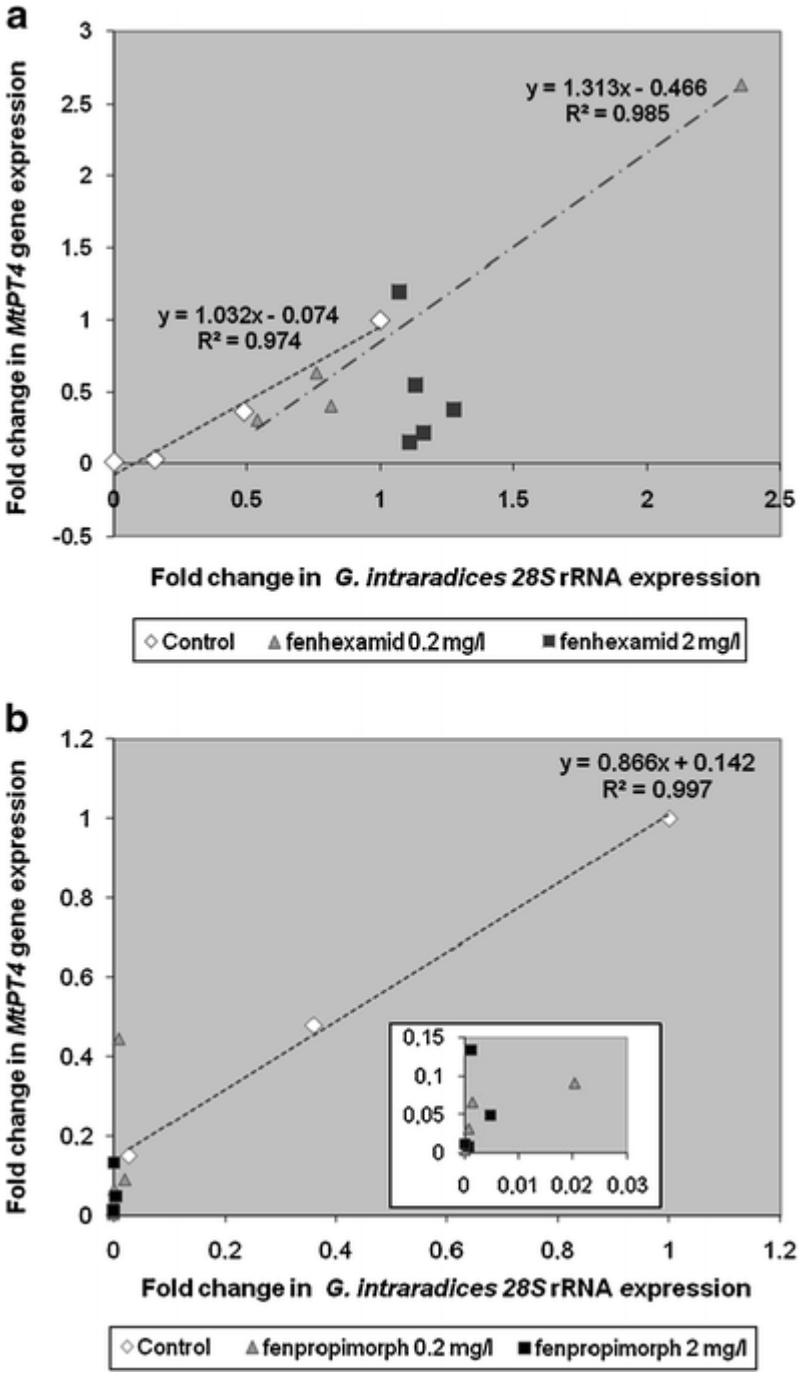

Fig. 2 Correlation between RNA expression levels of the *28S* ribosomal gene and *MtPT4* gene in roots of *M. truncatula* grown in association with the AM fungal strain MUCL 43204. The AM fungal extraradical mycelium developed in a hyphal compartment, in the absence (control) or presence of fenhexamid (**a**) or fenpropimorph (**b**). Each symbol (*rhombus*, *square*, *triangle*) represented a single plant. In each sample, the fold change in gene expression was calculated with reference to *M. truncatula GAPDH* transcript levels

Discussion

Fenpropimorph and fenhexamid are SBI molecules broadly used for the control of undesired fungal plant pathogens. However, their side-impact on non-target beneficial microorganisms such as AM fungi has rarely been considered. A number of recent investigations showed that fenpropimorph and fenhexamid have an impact on the growth of the AM fungus and the development of the AM symbiosis (Campagnac et al. 2008, 2009; Zocco et al. 2008). In particular, Zocco et al. (2008)

demonstrated that the extraradical mycelium network, supporting the branched absorbing structures, was impacted. This presumably could result in a decrease in the capacity of the AM fungus to take up, translocate and transport nutrients such as P. Here, *M. truncatula* plants were associated in vitro to an AM fungus, whose extraradical mycelium developed in a HC supplemented with fenpropimorph or fenhexamid. Transport of labelled P from the HC to the plant as well as the activity of ALP and SDH in the extraradical mycelium and the expression of the plant *MtPT4* gene was examined.

The transport of P was clearly observed in the untreated controls as well as in the presence of both SBI molecules and concentrations tested (i.e. 0.2 and 2 mg l^{-1}). P was measured, after 72 h, in hyphae, roots and shoots demonstrating the capacity of the AM fungus to take up, translocate and transport P from a root-free (i.e. the HC) compartment to its host plant even in the presence of SBI molecules. The translocation of P from the HC to the RC was exclusively via active mechanisms since no translocation of P was noted in the formaldehyde controls. However, marked differences were noted between the two SBI molecules. While no impact on P transport was noted in the presence of fenhexamid at the two concentrations tested, a significant decrease was observed with fenpropimorph, especially at the highest concentration. This observation may be largely due to the decrease in fungal biomass observed in the fenpropimorph treatment. Indeed in the presence of fenhexamid no impact was noted on hyphal weight and surface covered by the mycelium, while these parameters were drastically impacted by fenpropimorph at both concentrations. This corroborated earlier findings by Zocco et al. (2008) who observed a significant impact of fenpropimorph on fungal development at 0.2 and 2 mg l^{-1}, while no effect was observed with fenhexamid.

Considering the phosphorus activity per fungal biomass (Bq/µg mycelium), it seems that fenpropimorph does not impact on the capacity of the fungus to take up P. Interestingly, Ezawa et al. (2003) demonstrated that P uptake and subsequent polyP biosynthesis in AM fungi are surprisingly rapid and suggested that the rate-limiting step of the P delivery system is the translocation or transfer to the host.

The overall effect of fenpropimorph on P metabolism in the AM symbiosis seems therefore not on the fungal P uptake but mainly on P translocation. This fits with the gene expression data showing low MtPT4 transcript levels in mycorrhizal roots treated with the fungicide. In addition, it has been shown that fenpropimorph strongly affects sterol metabolism in the host plant (Campagnac et al. 2008): we might also speculate that this perturbation may in turn affect the activities occurring at the periabuscular membrane, including P uptake.

Enzymatic activity was measured as the proportion of extraradical hyphae exhibiting ALP and SDH activity. We observed a decrease of general metabolic activity, as highlighted by the proportion of hyphae exhibiting SDH activity for both SBI molecules. This decrease was significant with fenhexamid at both concentrations, while with fenpropimorph it was only significant at the highest concentration of the molecule (i.e. 2 mg l^{-1}). With both SBI molecules, the SDH activity remained above 75%. Similarly, the proportion of hyphae exhibiting ALP activity was reduced in the presence of both SBI molecules. However, this decrease was particularly drastic in the presence of fenpropimorph (less than 40% and 20% of hyphal length showed ALP activity in the presence of 0.2 and 2 mg l^{-1}, respectively, while the control exhibited close to 90% of hyphal length with ALP activity). In the presence of fenhexamid, only a small reduction in activity was observed and more than 90% of the hyphal length showed ALP activity whatever was the concentration of the fenhexamid tested (0.2 and 2 mg l^{-1}). The results indicated that fenpropimorph did strongly and specifically reduce the ALP activity of the AM fungus which could have clear implications on the AM fungal P metabolism and transport. Therefore, the decrease in P transport observed in the

presence of fenpropimorph may be related both to the decrease in fungal biomass and the inhibition of enzyme activity, especially ALP.

The ALP activity of extraradical mycelium was significantly reduced at both levels of fenpropimorph (at 0.017 and 0.17 times the recommended values for field application). Kjoller and Rosendahl (2000) applied the same molecule at one and 100 times the recommended field level and found an inhibition of ALP activity of extraradical mycelium only at the highest level. Schweiger and Jakobsen (1998), using a dose–response curve covering the range of 0.001 to 100 times the recommended field application in compartmented pot systems, did not measure a negative effect on P uptake by the extraradical mycelium. These differences could be attributed to several factors, among which are sorption and degradation of the fungicides. Adsorption processes are complex and yet not fully understood. Adsorption to soil particles and organic matter, sedimentation of particles (Braskerud and Haarstad 2003), soil pH, organic carbon content (Kah and Brown 2007) and soil moisture (Roy et al. 2009) are parameters that have been shown to influence the environmental fate, biological activity and bioavailability of SBI fungicides/molecules. Identically, even though degradation of fenpropimorph by bacteria has not been described yet, to our knowledge, it has been reported for other molecules such as propiconazole (Sarkar et al. 2009), mancozeb and thiophanate-methyl (Saha et al. 2008). Therefore, it is likely that results obtained with a soil substratum (Schweiger and Jakobsen 1998; Kjoller and Rosendahl 2000) may differ from those obtained on a gelled growth medium and that comparing recommended field doses in both conditions may yield divergent results. The in vitro culture systems therefore offer the advantage to circumscribe the environmental conditions to a limited number of variables to investigate more precisely the impact of fungicide molecules on the plant/fungus association, while pot experiments may sketch a more integrated picture of the impact of SBI fungicides/molecules on this association. Both approaches are therefore complementary.

The establishment of the AM symbiosis leads to a rearrangement of the Pi fluxes with the activation of specific PT genes. We monitored the expression levels of *MtPT4* which is exclusively expressed in arbuscule-containing cells and is essential for AM symbiotic Pi transport in *M. truncatula* (Javot et al. 2007b). The expression of this gene therefore represents one functional key feature of the AM symbiosis and provides important information about the symbiotic state of the colonized root system (Isayenkov et al. 2004). Fenhexamid applied to the HC of the AM-P in vitro culture systems did not strongly affect the development of the extraradical structures, neither the capability of the AM fungus to take up and transport Pi to the plant. This was also mirrored by the *MtPT4* expression levels which were similar between the SBI molecules-treated and control treatments. It is worth to note that a good correlation exists between *MtPT4* expression levels and the fungal 28S rRNA abundance for the control samples and the samples treated with the lowest concentration of fenhexamid. This is in line with the observation of Isayenkov et al. (2004). By contrast, the highest fenhexamid concentration led to a slight decrease of *MtPT4* expression levels notwithstanding that the fungal rRNA abundance was not affected. However, this decrease did not lead to a negative impact on ^{33}P translocation to the host plant. On the contrary, fenpropimorph highly affected the development of the AM fungus and its capacity to take up and translocate ^{33}P. Very low *MtPT4* expression levels were observed in treated samples and this was paralleled with the low abundance of fungal rRNA. Thus, in the fenpropimorph treatments, a discrepancy was observed between the colonization levels, as deduced from the microscopic observations and from the molecular analysis: the fungal rRNA was barely detected inside the roots. It can be hypothesized that the intraradical fungal structures were not metabolically active. It is worth to note that the number of active crossing hyphae from the RC to the HC, which is a measurement of the vitality of the AM fungus, was drastically reduced in the fenpropimorph treatment. Taken in their whole, the data on *MtPT4* for fenpropimorph are in agreement with the Pi translocation to the host plant being severely affected.

In this study, we reported for the first time the impact of two SBI molecules (fenpropimorph and fenhexamid) at two concentrations (0.2 and 2 mg l^{-1}) on the whole plant/AM fungus association under strict in vitro culture conditions. Previous investigations on transformed carrot roots showed that fenpropimorph appears more detrimental than fenhexamid not only on the AM fungus but also on the AM symbiosis (Campagnac et al. 2008). Here we demonstrated that both SBI molecules impacted the AM fungus, and again fenpropimorph appeared more toxic as reported earlier (Zocco et al. 2008; Campagnac et al. 2008). This was evidenced by either the decrease in P transport and ALP and SDH enzymatic activities measured in the extraradical mycelium or the *MtPT4* expression level as compared to the non-stressed plant/AM fungus association. These three parameters were intimately associated with the development of the AM fungus, suggesting a direct impact not only on the AM fungal development but also on the physiology and metabolism of the AM fungus. It is worth to note that an interference with sterol metabolism (Campagnac et al. 2009; Oger et al. 2009) and the induction of an oxidative stress (González-Guerrero et al. 2010) have been recently demonstrated.

The AM-P in vitro culture system used in our experiment appeared as a suitable alternative to pot experiments to investigate the mechanisms behind the impact of fungicide molecules on the non-target AM fungal symbionts. The system is reproducible, allowing different analysis, from radio-labelled P to enzymatic staining and molecular analysis, and the screening under strict controlled conditions of various actual/new candidate molecules having different targets in the sterol or other biosynthetic pathway. With this system, it was clearly shown that fenpropimorph and fenhexamid differently affected the physiology and metabolic activities of the AM fungus, suggesting that these molecules may differently impact on the sterol biosynthetic pathway in AM fungi.

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