Mycorrhiza (2009) 19:393–402
DOI 10.1007/s00572-009-0243-4

ORIGINAL PAPER

Development and activity of *Glomus intraradices* as affected by co-existence with *Glomus claroideum* in one root system

Martina Janoušková · Pascale Seddas · Libor Mrnka · Diederik van Tuinen · Anna Dvořáčková · Marie Tollot · Vivienne Gianinazzi-Pearson · Miroslav Vosátka · Armelle Gollotte

Received: 25 November 2008 / Accepted: 30 March 2009 / Published online: 18 April 2009
© Springer-Verlag 2009

Abstract The co-existence of two arbuscular mycorrhizal fungal (AMF) species, *Glomus intraradices* and *Glomus claroideum*, in the root systems of plants was investigated in a greenhouse experiment aimed at reconstructing interactions during an early stage of primary succession on a coal-mine spoil bank in Central Europe. Two plant species, *Tripleurospermum inodorum* and *Calamagrostis epigejos*, were inoculated either with one or both AMF species. Fungal development, determined by trypan blue and alkaline phosphatase staining as well as by PCR amplification of rRNA genes with species-specific primers, and the expression of five genes with different metabolic functions in the intraradical structures of *G. intraradices* were followed after 6 and 9 weeks of cultivation. The two AMF closely co-existed in the root systems of both plants possibly through similar colonisation rates and competitivity. Inoculation with the two fungi, however, did not bring any additional benefit to the host plants in comparison with single inoculation; moreover, plant growth depression observed after inoculation with *G. claroideum* persisted also in mixed inoculation. The expression of all the assayed *G. intraradices* genes was affected either by host plant or by co-inoculation with *G. claroideum*. The effects of both factors depended on the time of sampling, which underlines the importance of addressing this topic in time-course studies.

Keywords Alkaline phosphatase · Arbuscular mycorrhiza · Gene expression · Intraradical mycelium · Nested PCR

M. Janoušková (✉) · L. Mrnka · A. Dvořáčková · M. Vosátka
Institute of Botany, Academy of Sciences of the Czech Republic, 252 43 Průhonice, Czech Republic
e-mail: janouskova@ibot.cas.cz

P. Seddas · D. van Tuinen · M. Tollot · V. Gianinazzi-Pearson · A. Gollotte
UMR 1088 INRA/5184 CNRS/Université de Bourgogne, Plante-Microbe-Environnement,
BP 86510, 21065 Dijon Cedex, France

Introduction

In natural habitats, roots of arbuscular mycorrhizal plants are almost always colonised by a variety of arbuscular mycorrhizal fungal (AMF) species. AMF co-occurring on one locality differ in their root colonisation ability, effects on plant performance and compatibility with the plant species present (van der Heijden et al. 1998; Helgason et al. 2002). It has been suggested that the host plant benefits from the co-existence of several AMF species in its root system due to functional complementarity of the different species (Koide 2000). On the other hand, interspecific interactions between AMF may also result in a negative feedback on plant growth (Edathil et al. 1996; Violi et al. 2007).

When two or more AMF colonise one root system, the fungus that colonises most rapidly has the competitive advantage and can almost exclude the other fungi (Abbott and Robson 1983; Hepper et al. 1988). In contrast to previous assumptions, the competitive advantage is not related to prior occupancy of the root space, but has a physiological basis possibly involving carbohydrate supply by the host (Pearson et al. 1993; Vierheilig 2004). On the other hand, colonisation by certain AMF can be stimulated by the presence of other AMF species (van Tuinen et al. 1998a). The coexistence of AMF in one root system is also modified by the plant genotype (Alkan et al. 2006; Pivato et al. 2007), P availability (Alkan et al. 2006) and stress

factors such as the presence of heavy metal-spiked sewage sludge (Jacquot et al. 2000).

Determination of AMF colonisation based on a non-vital staining or PCR amplification of fungal DNA from root segments (van Tuinen et al. 1998a) gives an overview on the frequency of the fungal structures in the roots, but ignores their activity. Assessing fungal activity in roots may give another picture of the interaction between multiple species, as was already pointed out by Hepper et al. (1988). The physiological activity of intraradical fungal structures can be estimated using histochemical detection of fungal enzymatic activity. Among these, staining for alkaline phosphatase (ALP) has been proposed as an indicator of the activity of the phosphate metabolism because an increase in the frequency of ALP-active intraradical mycelium corresponded with a subsequent important increase in plant P content (Guillemin et al. 1995; Tisserant et al. 1996). In a more recent study, ALP genes of two AMF were identified and their expression was studied (Aono et al. 2004). The results of this study indicate that the ALP of AMF has a role in nutrient exchange with the host plant (Aono et al. 2004). Quantitative RT-PCR has been used to assess the activity of individual AMF species in roots by monitoring transcript accumulation of a β-tubulin gene (Rhody et al. 2003), rRNA (Delp et al. 2003) or genes associated with metabolic functions. Numerous AMF genes have been identified and the metabolic functions were characterised (Balestrini and Lanfranco 2006). Some of these may be useful markers for AMF activity or developmental stages. However, available information on the expression of AMF metabolic genes originates from systems consisting of one model plant colonised with one fungus and no information is available on how the metabolism of AMF is modulated in different plant species or how it is influenced by the coexistence of more than one AMF in a root system. It can be assumed that competitively weaker species are not only suppressed in their development but also in their activity. Furthermore, AMF species may react to competitors by accelerating or decelerating the development and turn-over of intraradical structures, which will be reflected by shifts in metabolic activities.

In the present study, we investigated the interaction of two AMF species, *Glomus intraradices* and *Glomus claroideum*, in one root system focusing on the development of root colonisation, ALP activity in the intraradical fungal mycelium and effects on plant growth and P uptake. Furthermore, we tested whether and to what extent the presence of *G. claroideum* affects *G. intraradices* at the level of expression of the ALP gene and of four other genes related to different metabolic processes: glutamine synthetase (GS) for nitrogen turn-over, superoxide dismutase (SOD) for anti-oxidative metabolism, stearoyl-coA desaturase (DS) for lipid turn-over and carboxypeptidase precursor (PEP) for protein turn-over. Although none of these four genes has been directly associated with a process involved in the interaction with the host plant, their expression is indicative of the metabolic activity of the intraradical fungal structures in general. The identity of the host plant is an important factor for the development of co-existing AMF species in the root system (Alkan et al. 2006). The experiment was therefore conducted with two host plant species, which should indicate whether particular results can be generalised. The plants, fungi and substrate used corresponded to the naturally occurring situation of an early stage of primary succession on a coal-mine spoil bank.

Material and methods

Material

The annual dicot *Tripleurospermum inodorum* (L.) Schultz-Bip. and the perennial grass *Calamagrostis epigejos* (L.) Roth were selected as ecologically distinct model representatives of plant communities colonising spoil banks of the Most coal basin (North-Bohemia, Czech Republic). *T. inodorum* is a member of the highly mycotrophic plant family of Asteraceae, while *C. epigejos* belongs to the C3 grasses, which are considered facultatively mycotrophic (Hetrick et al. 1990). They were cultivated in loess (pH 8.0; C_{org} 0.38%; C/N 1.54; P 24.8 mg kg^{-1}) collected from the freshly formed spoil bank of the Vršany coal mine. The field-collected substrate was sterilised by γ-irradiation (50 kGy). Four inoculation treatments were established for each plant species: (1) non-inoculated (=non-mycorrhizal) control; (2) inoculated with *G. intraradices* Schenck & Smith, isolate BEG140; (3) inoculated with *G. claroideum* Schenck & Smith, isolate BEG96; (4) inoculated with both isolates. The isolates originate from a man-made ecosystem (a pyrite smelter sedimentation pond near Chvaletice, Czech Republic) and have been previously proven to develop well in the spoil bank substrate used in the experiment (Püschel et al. 2007).

Establishment of the experiment and cultivation conditions

Seeds were germinated, and plantlets pre-grown in sand sterilised by autoclaving (20 min at 120°C). After 3 weeks, they were transferred into 400-ml pots with the culture substrate (1 plant per pot). Inoculation was performed with air-dried, homogenised substrate from 4-month-old cultures of the two AMF isolates (1:1 mixture of sand and zeolite), which contained colonised root fragments, mycelium and

spores. The mycorrhiza potential of both inocula had been tested in a dilution series prior to use: Presence and absence of mycorrhizal colonisation had been recorded in four replicate pots per dilution (inoculum/total amount of substrate—1:10, $1:10^2$, $1:10^3$, $1:10^4$) on *Trifolium repens* after 3 weeks. According to the results of this test, inoculum of *G. intraradices* had 10× higher colonisation potential than that of *G. claroideum*, and it was therefore diluted 1:10 with sand and zeolite to equalise the mycorrhizal potential of both inocula. Inoculation was performed with 25 g of inoculum of each fungus, i.e. the mixed-inoculation treatment received 50 g of inoculum in total. The non-inoculated treatments received inoculum inactivated by autoclaving. The inoculum was applied 2 cm below the plantlets. In order to equalise microbial conditions in the different inoculation treatments, all pots were irrigated with 10 ml of bacterial filtrate obtained by passing a suspension from the inocula through a filter paper (Whatman no. 1).

The plants were cultivated in a greenhouse (March–April) with light supplement (12 h, metalhalide lamps, 400 W); they were watered daily according to their needs. Each treatment consisted of 12 replicates: Six replicates were harvested after 6 weeks and six replicates after 9 weeks. The harvests were timed to cover expected peaks of ALP activity in the intraradical structures of *G. intraradices* when colonising the roots of *T. inodorum* (at 6 weeks) and *C. epigejos* (at 9 weeks). The expectation was based on a preliminary experiment, in which ALP activity was recorded after histochemical staining at four consecutive harvests.

Plant and mycorrhizal parameters recorded at harvest

Shoot biomass was determined after drying at 80°C for 24 h. P concentrations were determined spectrophotometrically at a wavelength of 630 nm (Unicam UV4-100) after digestion of the shoot biomass in 65% HNO_3 and 30% H_2O_2. From each plant, one root sample was stained with 0.05% trypan blue in lactoglycerol (Koske and Gemma 1989) to visualise all fungal structures in the roots. A second root sample was stained for ALP activity in the intraradical mycelium according to the protocol of Tisserant et al. (1993). The roots were cleared for 3 h in an enzyme solution (0.05 M Tris–HCl pH 9.2, 0.05% sorbitol, 15 U ml^{-1} cellulase and 15 U ml^{-1} pectinase), incubated overnight in the ALP staining solution (0.05 M Tris–HCl pH 9.2, 1 mg ml^{-1} Fast Blue RR salt, 1 mg ml^{-1} α-naphtyl acid phosphate monopotassium salt, 0.5 mg ml^{-1} $MgCl_2$ and 0.5 mg ml^{-1} $MnCl_2$) and incubated for 10 min in a sodium hypochlorite solution (5% active chlorine). Three parameters (F, M and A, see below) were calculated to quantify root colonisation and ALP activity in the samples according to Trouvelot et al. (1986). Briefly, from each stained root subsample, 30 segments of 1 cm were mounted onto microscopic slides, and every segment was assigned to one of six classes of intensity of mycorrhizal infection or ALP activity and to one of four classes of abundance of arbuscules, total or ALP active. F (frequency in the root system) was calculated as percentage of root segments with at least a trace of fungal structures or ALP activity. M (intensity of colonisation of the root system) and A (abundance of arbuscules in the root system) were calculated based on the frequency of the segments in the classes by the formulas provided by Trouvelot et al. (1986) using the programme "Mycocalc" (http:/www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

Quantification of AMF isolates in the mixed-inoculation treatment

In the treatments with dual inoculation, fungal frequency was quantified by 25S rDNA-targeted nested PCR (van Tuinen et al. 1998a). The first step was performed with the eukaryotic primer pair LR1 and NDL22 (van Tuinen et al. 1998b). Three second-step PCRs were performed from each product of the first step: (1) with AMF-general primer pair FLR3 and FLR4 (Gollotte et al. 2004) as positive control, (2) and (3) with two primer pairs newly designed to discriminate the inoculated species *G. intraradices* (for: 5′ GAT TGA AGC CAG TCG TAC CT 3′; rev: 5′ CGT TCT AAC CTA TTG ACC ATC 3′) and *G. claroideum* (for: 5′ ATT GAA GTC AGT CGT GCT GG 3′; rev: 5′ ACC AAA GAG AAG CCA GGT G 3′).

One-centimetre root segments were randomly picked from the root system and air-dried in Eppendorf tubes (1.5 ml). For DNA extraction, they were crushed in 40 µl of TE buffer (pH 8.0) and 10 µl of 20% suspension of Chelex®100 (Bio-Rad; *C. epigejos*) or in 50 µl of TE buffer with charcoal and polyvinylpolypyrrolidone (Sigma) added (*T. inodorum*). The crude extract was heated (95°C, 10 min), centrifuged (12,000 rpm, 5 min), diluted 1:10 and added into the first-step PCR reaction in the amount of 5 µl. The product of the first-step PCR was diluted 1:500 and added in the amount of 5 µl into the second-step reactions. PCRs were performed in the final volume of 20 µl with the following concentrations of ingredients: 1× concentrated PCR buffer, 1.5 mM $MgCl_2$, 200 nM dNTP, 500 nM each primer, 1 U Taq polymerase (MBI Fermentas). After a 4-min denaturation at 93°C, 30 cycles (first-step reaction) or 25 cycles (second-step reaction) were performed with 93°C (1 min), 58°C (1 min) and 72°C (1 min), followed by 10 min final extension at 72°C. The expected length of the PCR products was confirmed by electrophoresis in 1% (*w*/*v*) denaturing agarose gel. From the first harvest, the presence

or absence of each AMF species was recorded in 15 root segments per plant on four replicate plants per plant species. From the second harvest, only five root segments per three replicate plants were analysed because all mycorrhizal segments contained both fungi.

Fungal gene expression profiling

At each harvest, three plants per treatment were randomly selected from the treatments, which were inoculated with *G. intraradices* only or with both fungi, and one plant per treatment was checked from the treatments, which were left non-inoculated or inoculated with *G. claroideum* only. Total RNA was extracted from each plant root system according to the method of Franken and Gnädinger (1994), modified as described by Weidmann et al. (2004). RNA quantity and quality were estimated by absorbance at 260 and 280 nm and by electrophoresis in 1% (*w*/*v*) denaturing agarose gel. cDNA was prepared from 1 μg total DNAseI-treated (Promega, Madison, USA) RNA using 1.5 μg of oligodT_{15}, 300 U MMLV reverse transcriptase (EC 2.7.7.49) and 80 U RNase inhibitor during 15 min at 25°C followed by 1 h at 42°C and 2 min at 72°C, as described by Weidmann et al. (2004). Successful cDNA synthesis was confirmed by PCR amplification of the constitutively expressed plant GAPDH gene. The following primer pairs deduced from EST sequences were tested by ordinary PCR whether they amplify specifically from *G. intraradices* cDNA but not from *G. claroideum* cDNA or from non-mycorrhizal roots: GS (for: 5′ CGT GGT GGT GAT AAT ATC C 3′; rev: 5′ CGC ACC AAC AGA ACA ATA AT 3′); SOD (for: 5′ CTG GAC CTC ATT TTA ACC CA 3′; rev: 5′ CCG ATA ACA CCA CAA GCA A 3′); DS (for: 5′ TCG TGT TCC TGA AAA TGA AG 3′; rev: 5′ GCT TTA GTG GAG TCT TTA CC 3′), PEP (for: 5′ GCA GCA GAA GAT ACA AAA TG 3′; rev: 5′ CTT ATG ACC AAG CGT CAG 3′), ALP (for: 5′ ATA GCC TTG TCA CTG ATT CC 3′; rev: 5′ GTT TGG CAG ATT CCA ACA CG 3′) and the constitutively expressed alpha tubulin used as reference (for: 5′ CGG TGA AGG TAT GGA AGA AG 3′; rev: 5′ GGC CAC CAA GTC ACG AAA A 3′). *G. intraradices* transcript accumulation was monitored by real-time RT-PCR as described by Seddas et al. (2009). The gene-specific cDNA fragments were amplified using the QuantiTect™ SYBR® GreenPCR mix (Qiagen, Hilden, Germany) and the above specified primers. cDNA from non-mycorrhizal roots and from roots colonised by *G. claroideum* only were run as negative controls. Fluorescence was quantified using an ABI PRISM 7900 apparatus (Applied BioSystems, Foster City, CA, USA).

The expression of each gene in each plant was assayed in duplicate in a volume of 25 μl containing 1× SYBR green reagent and 1 μM each gene-specific primer pair. After 10 min denaturation at 95°C, 40 PCR cycles were performed as recommended by the manufacturer (95°C during 15 s, the appropriate primer annealing temperature during 30 s and 72°C during 30 s). To verify amplification of each specific target cDNA, a melting-curve analysis was included at the end of each PCR run according to the thermal profile suggested by the manufacturer (95°C during 15 s, the appropriate primer annealing temperature during 15 s and 95°C during 15 s). The generated data were analysed by SDS 2.2 software (Applied Biosystems). For all amplification plots, the baseline data were set with the automatic C_T function available with SDS 2.2 by calculating the optimal baseline range and threshold values by using the AutoC_T algorithm (SDS 2.2 User Manual). Candidate-gene expression data from real-time RT-PCR were plotted as $2^{(40-CT)}/10$, as described in Czechowski et al. (2004), and normalised against the constitutively expressed alpha tubulin gene.

Statistical analyses

Data on mycorrhizal colonisation, shoot dry weight and P content were analysed by three-way ANOVA with the fixed factors plant, harvest and inoculation. Comparisons among means were carried out using Tukey's multiple comparison test at significance level $P<0.05$ using the SPSS 15.0 software. Data on transcript levels led to unbalanced design; the GLM ANOVA with all three factors (fungi, plants, harvest) fixed was therefore applied for their analysis (SAS 8.2 software).

Results

Root colonisation and ALP activity of intraradical fungal structures

Frequency (F) and intensity (M) of root colonisation was significantly higher in *T. inodorum* than in *C. epigejos* at both harvests and increased in both plants in the order *G. claroideum*≤*G. intraradices*≤dual inoculation (Table 1). Similar differences between treatments were also obtained for the abundance of arbuscules (data not shown). The frequency of ALP-active intraradical fungal structures was relatively high at both harvests and was significantly higher at the first harvest (71–98%) than at the second harvest (54–90%; Table 1). The intensity of root colonisation by ALP-active fungus (Table 1) and the abundance of ALP-active arbuscules (data not shown) did not differ between harvests, and differences between treatments followed similar trends as described above for root colonisation.

The frequency of total root colonisation determined by PCR from root segments was lower than the frequency determined by trypan blue staining at the first harvest (85%

Table 1 Frequency (F) and intensity (M) of root colonisation and alkaline phosphatase (ALP) activity of fungal structures in the roots of *Calamagrostis epigejos* (CE) and *Tripleurospermum inodorum* (TI) at two harvests, after 6 and 9 weeks of cultivation

Plant	Inoculation	Colonisation F (%)		Colonisation M (%)		ALP activity F (%)		ALP activity M (%)	
		6 weeks	9 weeks	6 weeks	9 weeks	6 weeks	9 weeks	6 weeks	9 weeks
CE	GC	79 (8) c	75 (14) z	33 (8) c	26 (8) z	71 (14) b	54 (23) z	12 (10) c	11 (8) z
	GI	92 (6) b	87 (9) yz	37 (6) c	36 (10) yz	72 (11) b	68 (4) yz	22 (6) c	16 (8) z
	GC+GI	97 (2) a	96 (6) x	49 (8) b	43 (6) y	94 (6) a	86 (8) xy	23 (6) c	22 (9) yz
TI	GC	90 (8) b	94 (7) xy	53 (8) b	59 (14) x	73 (5) b	76 (26) xy	16 (5) c	36 (20) xy
	GI	99 (2) a	98 (3) x	86 (5) a	85 (6) w	96 (4) a	90 (7) x	50 (10) b	43 (16) x
	GC+GI	99 (1) a	99 (2) x	81 (7) a	84 (7) w	98 (4) a	89 (11) x	71 (10) a	39 (13) xy
Significance (*F* value)									
Harvest (A)		n.s. (0.3)		n.s. (0.0)		* (6.8)		n.s. (3.4)	
Plant (B)		*** (41.1)		*** (359.5)		*** (24.4)		*** (90.1)	
Inoculation (C)		*** (27.5)		*** (46.7)		*** (23.1)		*** (21.4)	
A×B		n.s. (0.8)		n.s. (3.4)		n.s. (0.4)		n.s. (1.0)	
A×C		n.s. (0.7)		n.s. (0.0)		n.s. (1.3)		*** (9.6)	
B×C		n.s. (2.0)		*** (15.6)		* (3.8)		* (4.9)	
A×B×C		n.s. (0.3)		n.s. (0.8)		n.s. (1.8)		*** (10.0)	

Data are means of six replicates (SD). Values within each harvest marked by the same letter are not significantly different ($P<0.05$; Tukey's multiple range test). Effects of factors according to three-way ANOVA: n.s. non-significant effect; *$P<0.05$; ***$P<0.001$. Inoculation: GC=*Glomus claroideum*, GI=*Glomus intraradices*

vs. 99% in *T. inodorum*, 82% vs. 97% in *C. epigejos*). At the second harvest, the values were similar (100% vs. 99% in *T. inodorum*, 87% vs. 96% in *C. epigejos*). Root segments, from which an amplification product was obtained with the AMF primer pair FLR3/FLR4, also always gave an amplification product with at least one of the species-discriminating primer pairs. More than half the colonised root segments were occupied by both fungal species at the first harvest (Fig. 1). At the second harvest, both fungi occupied 100% of the colonised root segments.

Mycorrhizal effects on plant growth and P uptake

Mycorrhizal effects on plant growth were similar in *T. inodorum* and *C. epigejos* and consistent in both harvests (Table 2, no significant interactions). *G. intraradices* alone did not have any effect on shoot biomass, while *G. claroideum* alone or dual colonisation mostly decreased shoot growth. The biomass of the plants colonised by both isolates was either similar or significantly lower than that of plants colonised by *G. intraradices* alone.

In contrast, mycorrhizal effects on P concentration in shoots depended partly on the plant species (Table 2): *G. claroideum* alone increased the P concentration in *C. epigejos* but had no effect on that in *T. inodorum*. *G. intraradices* alone and dual colonisation increased the P concentration in both plant species, but the effect of dual inoculation was more pronounced in *T. inodorum*. It had higher P concentration when colonised with both fungi than when colonised with *G. intraradices* alone, while there was no differences in P concentration between these two inoculation treatments in *C. epigejos*.

Expression of fungal genes

As expected, no amplification products were obtained from roots colonised by *G. claroideum* with any of the *G. intraradices* primer pairs, confirming their suitability for monitoring *G. intraradices* gene activity in roots colonised by both AMF. Significant effects of plant species, harvest time and fungal inoculation on relative transcript levels for the five genes are summarised in Table 3. As only

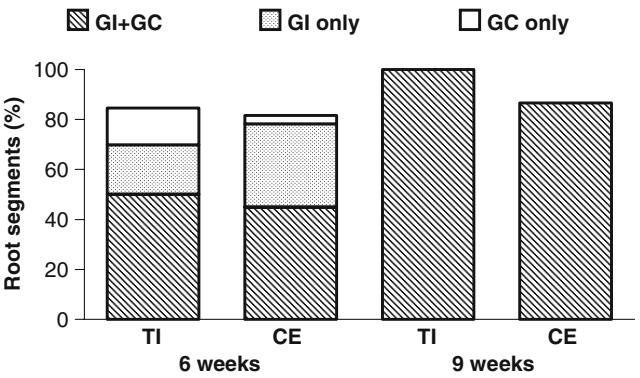

Fig. 1 Occupancy of root segments of *T. inodorum* (*TI*) and *C. epigejos* (*CE*) by *G. intraradices* (*GI*) and *G. claroideum* (*GC*) after dual inoculation as determined by PCR with discriminating primers after 6 and 9 weeks of cultivation

Table 2 Mycorrhizal effects on the shoot biomass and P content in the shoots of *Calamagrostis epigejos* (CE) and *Tripleurospermum inodorum* (TI) at two harvests, after 6 and 9 weeks of cultivation

Plant	Inoculation	Dry weight shoots (g)		P concentration in shoots (μg g^{-1})	
		6 weeks	9 weeks	6 weeks	9 weeks
CE	NI	0.18 (0.03) a	0.30 (0.03) k	2126 (486) b	1348 (244) l
	GC	0.14 (0.02) b	0.23 (0.03) kl	3484 (941) a	2219 (908) k
	GI	0.18 (0.03) a	0.25 (0.06) kl	3316 (1028) a	2560 (527) k
	GC+GI	0.13 (0.02) b	0.20 (0.08) l	2715 (448) ab	2443 (499) k
TI	NI	0.13 (0.05) p	0.21 (0.07) x	2434 (1044) r	2162 (712) z
	GC	0.09 (0.03) qr	0.19 (0.03) x	1895 (607) r	1653 (795) z
	GI	0.12 (0.02) pq	0.23 (0.03) x	4869 (789) q	3589 (718) y
	GC+GI	0.09 (0.01) r	0.19 (0.06) x	6466 (783) p	4916 (296) x
Significance (*F* value)					
Harvest (A)		*** (108.7)		*** (19.6)	
Plant (B)		*** (27.1)		*** (14.4)	
Inoculation (C)		*** (8.9)		*** (32.3)	
A×B		n.s. (0.6)		n.s. (0.6)	
A×C		n.s. (0.1)		n.s. (0.3)	
B×C		n.s. (1.3)		*** (18.6)	
A×B×C		n.s. (1.1)		n.s. (1.0)	

Data are means of six replicates (SD). Values within each combination of plant and harvest marked by the same letter are not significantly different ($P<0.05$; Tukey's multiple range test). Effects of factors according to three-way ANOVA: n.s. non-significant effect, ***$P<0.001$. Inoculation: NI=not inoculated, GC=*Glomus claroideum*, GI=*Glomus intraradices*

inoculation with *G. intraradices* and dual inoculation were compared from the four inoculation treatments, the factor inoculation corresponds to the effect of the presence of *G. claroideum* on the gene expression of *G. intraradices* in dually colonised plants.

Interactions between the factors plant species and inoculation were non-significant for all the fungal genes, meaning that the effects of *G. claroideum* on the gene expression in *G. intraradices* were similar in both plants. For all genes, however, the harvest time had a significant interaction with either plant species or inoculation, i.e. differences between plants or effects of the presence of *G. claroideum* depended on the age of the mycorrhiza. For this reason, the effects of the plant and inoculation factors were also determined for each harvest separately (Table 3).

The most pronounced differences between treatments were recorded in GS expression. Transcript levels of this gene sharply declined between the first and second harvest (Fig. 2a). At the first harvest, they were higher in *T. inodorum* than in *C. epigejos* mycorrhizal roots, while at the second harvest, they were almost undetectable in both plants. Other significant effects of the factors plant and inoculation were less pronounced, and they were not always reflected by differences between the treatments in

Table 3 Effects of the factors plant species (=Plant), co-inoculation with *G. claroideum* (=Inoculation) and age of the mycorrhiza (=Harvest) on the transcript levels of five *G. intraradices* genes in mycorrhizal roots according to three-way ANOVA (*F* values in parentheses)

	GS	SOD	DS	PEP	ALP
Inoculation (A)	n.s. (2.3)	n.s. (3.7)	n.s. (2.1)	** (9.0)	n.s. (2.5)
Plant (B)	*** (26.1)	n.s. (1.1)	* (8.3)	* (5.8)	* (5.3)
Harvest (C)	*** (61.9)	n.s. (1.5)	n.s. (0.4)	n.s. (2.3)	** (9.7)
A×B	n.s. (2.6)	n.s. (0.2)	n.s. (0.1)	n.s. (1.3)	n.s. (4.3)
A×C	n.s. (1.3)	n.s. (0.2)	n.s. (1.5)	* (6.3)	** (11.7)
B×C	*** (22.4)	* (6.0)	** (8.7)	n.s. (2.3)	n.s. (1.2)
A×B×C	n.s. (4.0)	n.s. (0.7)	n.s. (0.1)	n.s. (0.0)	n.s. (2.6)
Harvest after 6 weeks					
Inoculation (A)	n.s. (1.8)	n.s. (3.0)	n.s. (2.6)	** (12.2)	* (7.1)
Plant (B)	** (25.1)	n.s. (1.1)	n.s. (0.0)	n.s. (0.3)	n.s. (3.3)
A×B	n.s. (3.4)	n.s. (0.1)	n.s. (0.0)	n.s. (0.5)	n.s. (3.8)
Harvest after 9 weeks					
Inoculation (A)	n.s. (1.0)	n.s. (1.0)	n.s. (0.0)	n.s. (0.1)	* (6.9)
Plant (B)	n.s. (1.0)	* (5.5)	** (27.4)	* (10.1)	n.s. (3.0)
A×B	n.s. (1.0)	n.s. (0.7)	n.s. (0.3)	n.s. (0.9)	n.s. (0.4)

In the lower part of the table, the effects of the factors plant species and co-inoculation are given separately for each harvest. n.s. non-significant effect, *$P<0.05$, **$P<0.01$, ***$P<0.001$. For abbreviations of the genes, see Fig. 2

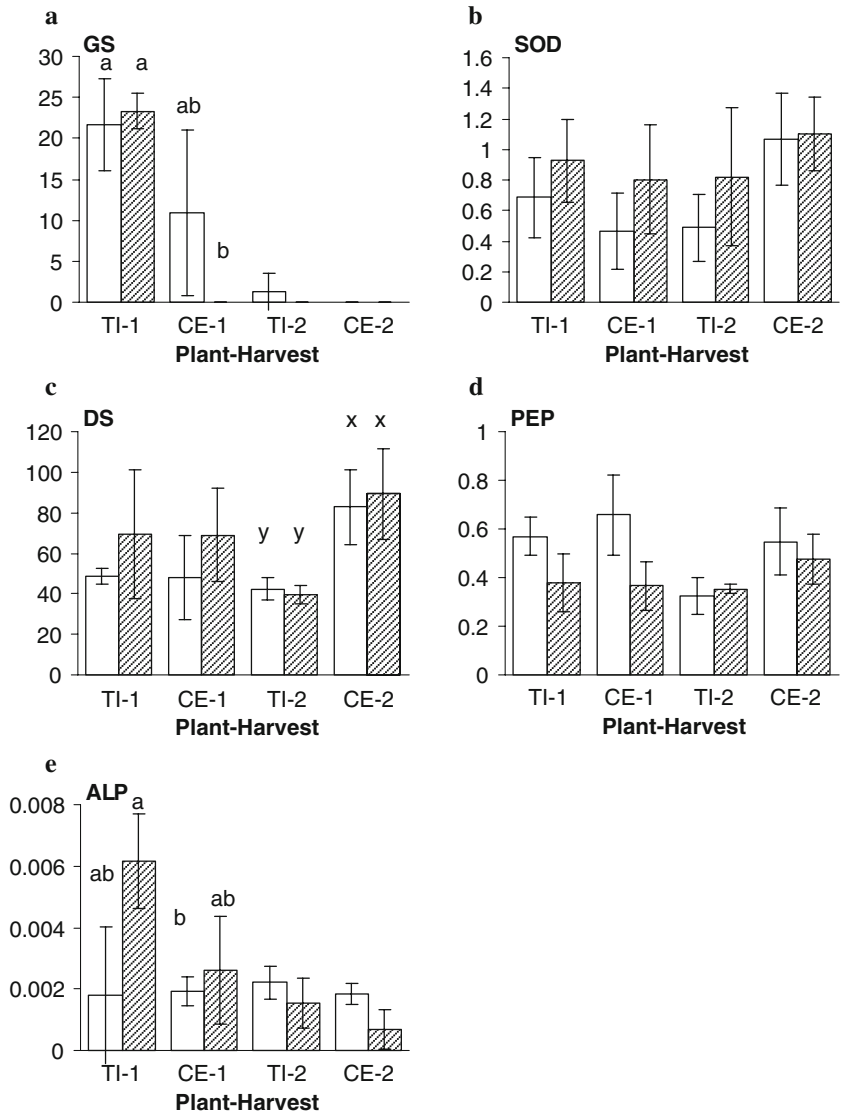

Fig. 2 Expression of glutamine synthetase (**a**, *GS*), superoxide dismutase (**b**, *SOD*), stearoyl-coA desaturase (**c**, *DS*), carboxypeptidase precursor (**d**, *PEP*) and alkaline phosphatase (**e**, *ALP*) in the intraradical structures of *G. intraradice*s BEG140 colonising the roots of *T. inodorum* (*TI*) or *C. epigejos* (*CE*), alone (*empty bars*) or together with *G. claroideum* (*dashed bars*). The gene expression is expressed as amount of mRNA normalised against the constitutively expressed alpha tubulin gene; columns are means of three replicates (SD). Values within each harvest are marked by *letters* when treatments were significantly different according to Tukey's multiple range test ($P<0.05$): Values not marked by the same *letters* are significantly different

multiple comparison tests. The three genes coding SOD, DS and PEP were more highly expressed in *C. epigejos* than in *T. inodorum* mycorrhizal roots at the second harvest (Table 3, Fig. 2b, c, d). Significant differences between inoculation treatments were limited to PEP and ALP. The expression of PEP was higher in *G. intraradices* alone than in the presence of *G. claroideum* at the first harvest (Table 3, Fig. 2d). The expression of ALP was higher in the presence of *G. claroideum* at the first harvest and vice versa at the second harvest (Table 3, Fig. 2e). ALP expression remained stable between the two harvests when *G. intraradices* was inoculated alone, but it decreased in the presence of *G. claroideum*, especially in *T. inodorum*. The means of ALP expression per treatment correlated with means of intensity (M) of histochemical ALP staining (Pearson correlation=0.81, $P<0.05$), but correlation of the single replicates was non-significant.

Discussion

Mycorrhizal colonisation was of similar frequency and intensity at both harvests in *C. epigejos* and *T. inodorum*, which shows that it had reached its plateau before 6 weeks

growth. The high proportion of root segments occupied by both *G. claroideum* and *G. intraradices* confirms that no spatial exclusion occurred between the two fungi, in contrast to observations of Jacquot-Plumey et al. (2001) on a community of five AMF morphotypes. It can be assumed that the two fungi colonised simultaneously similar root tissues, as reported by Cordier et al. (1996). Plants inoculated with the two fungi had the same or only slightly higher levels of root colonisation, as compared to plants inoculated with either alone. This suggests that either one or both fungi formed fewer intraradical structures in the presence of the other fungus, in accordance with the observations of Wilson and Trinick (1983) and Wilson (1984).

In the study of Jansa et al. (2008), the quantity of co-inoculated *G. intraradices* and *G. claroideum* in the roots of two plant species, medic and leek, was similar after 4 weeks of cultivation, but *G. intraradices* became dominant after 8 weeks. Our results, however, cannot be directly compared with those of Jansa et al. (2008) because their approach of quantification, quantitative real-time PCR, reflected to an unknown degree also the vitality of the fungi. In contrast, our approach, determination of the frequency of the fungi in root segments by ordinary PCR, is much less influenced by the vitality of the intraradical structures, and the results are comparable to those obtained by evaluating the frequency of AMF in roots after a non-vital staining. The latter approach was well capable to show, e.g. the depression of the spread of some AMF species/morphotypes by soil contamination in previous studies (Jacquot et al. 2000; Jacquot-Plumey et al. 2001) so we can conclude that none of the fungi was out competed by the other in this experiment.

The interaction between different AMF species in one root system depends on the rate of their spread: Faster root colonisation may bring competitive advantage (Abbott and Robson 1983; Hepper et al. 1988; Jansa et al. 2008). The competitive abilities of AMF can therefore also be influenced by the mycorrhizal potentials of their inocula, although Alkan et al. (2006) showed that equal mycorrhizal potential of co-inoculated fungi does not ensure their balanced development in the roots. In our experiment, the inocula of *G. claroideum* and *G. intraradices* had been standardised to the same mycorrhizal potential, i.e. both fungi colonised the plant roots at a similar rate in the early stage of mycorrhiza development. This no doubt contributed to the close co-existence of both fungi in the root systems of their hosts. In addition, our results also point to the existence of an interactive compatibility between *G. claroideum* and *G. intraradices*.

The absence of a positive growth response to inoculation in spite of an improved P uptake of plants colonised by *G. intraradices* or both fungi indicates that factor(s) other than P shortage must have been limiting mycorrhizal plant growth responses under the experimental conditions. Püschel et al. (2007) reported positive mycorrhizal growth effects on clover, *C. epigejos* and *T. inodorum* inoculated with the same isolates in very similar soil and light conditions. Poor light, often responsible for the absence of positive growth responses in mycorrhizal plants (Smith and Read 1997), could therefore not be the (only) factor responsible. The experiment by Püschel et al. (2007) was performed in larger microcosms, where AMF extraradical hyphae had more space to extend beyond the rooting zone of the plants. Zhu et al. (2001) suggested that small rooting volumes and high root densities make the exploration of the soil volume by extraradical hyphae less significant for plant acquisition of immobile nutrients.

The two fungal species partly differed in their effects on plant growth and P uptake, and the differential effects were maintained in dual colonisation. Plants colonised with both fungi displayed similar growth depression as when colonised with *G. claroideum* alone, and *T. inodorum* had higher P concentrations as when colonised with *G. intraradices* alone. The dual colonisation increased P concentrations in *T. inodorum* significantly more than colonisation with *G. intraradices* alone. However, this should not be interpreted as synergistic effect of the two fungi on P uptake as the dually colonised plants were smaller (presumably as effect of *G. claroideum*), and the total P uptake (P content per plant) did not significantly differ between the two inoculation treatments.

We can therefore conclude that the simultaneous colonisation by these two particular fungi did not have any synergistic effect in terms of plant growth promotion or enhancement of P uptake, in contrast to the results of Jansa et al. (2008) who used other isolates of the same fungal species. Jansa et al. (2008) suggested that the lack of synergistic effects of colonisation with multiple species, often reported in experiments, may be caused by the fact that one species becomes dominant and outcompetes the other co-inoculated species. This was, however, not the case in our experiment, and it is probable that the two isolates selected for the study did not complement in any function, in contrast to the *G. intraradices* and *G. claroideum* isolates used by Jansa et al. (2008). The ability to promote plant growth can vary greatly among isolates of one AMF species (Munkvold et al. 2004). In the conditions of our experiment, *G. claroideum* was the less effective symbiont especially with *T. inodorum*, suggesting that inoculating plants with a mixture of isolates may not bring any advantage over inoculation with a single isolate as the negative effects of an ineffective isolate may persist in a mixed inoculation.

Our study did not support the assumption that ALP activity could serve as an efficiency marker of the symbiosis (Tisserant et al. 1993; Guillemin et al. 1995). The *G. intraradices* and *G. claroideum* isolates differed in

their effect on P uptake of *T. inodorum*, but this was not accompanied by parallel differences in ALP activity in roots. This agrees with the conclusion of Boddington and Dodd (1998) who did not find any correlation between ALP activity in the intraradical mycelium of two AMF and increased P concentrations in the host plant. Based on the results of a preliminary experiment, the histochemically detected ALP activity in the intraradical fungal structures was expected to decline in *T. inodorum* and to rise in C. *epigejos* between the sixth and ninth week of cultivation, when *G. intraradices* was the only colonising fungus. However, it remained stable in both plant species. This indicates that both harvests took place before ALP activity started to decline as observed in aging cultures (Tisserant et al. 1993, 1996). When roots of *T. inodorum* were colonised by both AMF, the histochemically detected ALP activity in fungal structures decreased between the two harvests. The expression of the ALP gene in *G. intraradices* followed a similar trend remaining stable between the two harvests when *G. intraradices* was the only colonising fungus and declining in the mixed inoculation. This is in line with the conclusion of Aono et al. (2004) that the pattern of expression of the ALP gene in different life stages of AMF can be related to the pattern of ALP activity monitored by histochemical staining. The differences between the inoculation treatments may be related to the fact that ALP activity of AMF mycelium decreases with the age of the mycorrhizal association (Tisserant et al. 1993; 1996), and the dynamics may be affected by the co-existence of two fungi in one root system.

This is the first application of fungal marker genes to evaluate the influence of host plant, harvest time and inoculation treatment on compatibility between AMF colonising a same root system. Although results must be regarded as preliminary, the data do indicate differences in expression profiles between the five *G. intraradices* genes in that GS transcript levels vary both in time and with host plant, whilst SOD, ALP and DS transcripts tend to increase or be constant after co-inoculation with *G. claroideum*, and PEP tends to decrease in this treatment. Interestingly, the effects of co-inoculation depended on time but had the same tendency with the two host plant species. It would be interesting to follow the time-course expression of these and other genes within the intraradical fungal structures in more detail, as it could be related to the turn-over of these structures. In this particular experiment, differences in fungal development between harvests, plant and inoculation treatments were unexpectedly small, which does not enable to relate the differences to a particular developmental stage.

In conclusion, this study represents a first attempt to explore the effect of the co-existence of AMF on their activity by studying gene expression in the intraradical mycelium. It confirms that two species can closely co-exist in one root system, possibly through similar colonisation rates and compatible competitivity, even if this did not result in positive effects on plant growth or P uptake. The level of expression of the tested fungal genes changed with time, which underlines the importance of conducting such investigations as time-course studies with several samplings, and depended partly on the host plant. However, it has also been shown that the expression of some fungal genes is modulated by host plant and the coexistence with another AMF, and these changes should be further explored in order to understand the finely tuned physiological bases of mycorrhizal effects.

Acknowledgements The authors are grateful to Dr. Judith Fehrer for the design of discriminating primers for the quantification of AMF in roots and valuable advice to this part of the work. We would also like to thank to Dr. Jana Rydlová and Dr. David Püschel for providing plant seeds and to Barbora Slouková for technical support. Financial support for this study was provided by the Ministry of Education, Youth and Sports of the Czech Republic (Grant 1 M0571 and 2005-06-059) and by the Grant Agency of the Academy of Sciences of the Czech Republic (Grant AV0Z60050516).

References

Abbott LK, Robson AD (1983) Introduction of vesicular arbuscular mycorrhizal fungi into agricultural soils. Aust J Agric Res 34:741–749. doi:10.1071/AR9830741

Alkan N, Gadkar V, Yarden O, Kapulnik Y (2006) Analysis of quantitative interactions between two species of arbuscular mycorrhizal fungi, *Glomus mosseae* and *G. intraradices*, by real time PCR. Appl Environ Microbiol 72:4192–4199. doi:10.1128/AEM.02889-05

Aono T, Maldonado-Mendoza IE, Dewbre GR, Harrison MJ, Saito M (2004) Expression of alkaline phosphatase genes in arbuscular mycorrhizas. New Phytol 162:525–534. doi:10.1111/j.1469-8137.2004.01041.x

Balestrini R, Lanfranco L (2006) Fungal and plant gene expression in arbuscular mycorrhizal symbiosis. Mycorrhiza 16:509–524. doi:10.1007/s00572-006-0069-2

Boddington CL, Dodd JC (1998) A comparison of the development and metabolic activity of mycorrhizas formed by arbuscular mycorrhizal fungi from different genera on two tropical forage legumes. Mycorrhiza 8:149–157. doi:10.1007/s005720050228

Cordier C, Gianinazzi-Pearason V, Gianinazzi S (1996) An immunological approach for the study of spatial relationships between arbuscular mycorrhizal fungi in planta. In: Azcon-Aguilar C, Barea JM (eds) Mycorrhizas in integrated systems: from genes to plant development. European Commission, EUR 16758, Luxemburg, pp 189–194

Czechowski T, Bari RP, Stitt M, Scheible WR, Udvardi MK (2004) Real-time RT-PCR profiling of over 1400 Arabidopsis transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. Plant J 38:366–379. doi:10.1111/j.1365-313X.2004.02051.x

Delp G, Timonen S, Rosewarne GM, Barker SJ, Smith S (2003) Differential expression of *Glomus intraradices* genes in external mycelium and mycorrhizal roots of tomato and barley. Mycol Res 107:1083–1093. doi:10.1017/S0953756203008311

Edathil TT, Manian S, Udaiyan K (1996) Interaction of multiple VAM fungal species on root colonization, plant growth and nutrient status of tomato seedlings (*Lycopersicon esculentum* Mill). Agric Ecosyst Environ 59:63–68. doi:10.1016/0167-8809(96)0104-7

Franken P, Gnädinger F (1994) Analysis of parsley arbuscular endomycorrhiza-infection development and messenger-RNA levels of defense-related genes. Mol Plant Microbe Interact 7:612–620. doi:10.1094/MPMI-7-0612

Gollotte A, van Tuinen D, Atkinson D (2004) Diversity of arbuscular mycorrhizal fungi colonising roots of the grass species *Agrostis capillaris* and *Lolium perenne* in a field experiment. Mycorrhiza 14:111–117. doi:10.1007/s00572-003-0244-7

Guillemin JP, Orozco MO, Gianinazzi-Pearson V, Gianinazzi S (1995) Influence of phosphate fertilization on fungal alkaline-phosphatase and succinate-dehydrogenase activities in arbuscular mycorrhiza of soybean and pineapple. Agric Ecosyst Environ 53:63–69. doi:10.1016/0167-8809(94)00555-S

Helgason T, Merryweather JW, Denison J, Wilson P, Young JPW, Fitter AH (2002) Selectivity and functional diversity in arbuscular mycorrhizas of co-occurring fungi and plants from a temperate deciduous woodland. J Ecol 90:371–384. doi:10.1046/j.1365-2745.2001.00674.x

Hepper CM, Azcon-Aquilar C, Rosendahl S, Sen R (1988) Competition between three species of *Glomus* used as spatially separated introduced and indigenous mycorrhizal inocula for leek (*Allium porrum* L.). New Phytol 110:207–215. doi:10.1111/j.1469-8137.1988.tb00254.x

Hetrick BAD, Wilson GWT, Todd TC (1990) Differential responses of C3 and C4 grasses to mycorrhizal symbiosis, phosphorus fertilization, and soil microorganisms. Can J Bot 68:461–467. doi:10.1139/b90-061

Jacquot E, van Tuinen D, Gianinazzi S, Gianinazzi-Pearson V (2000) Monitoring species of arbuscular mycorrhizal fungi in planta and in soil by nested PCR application to the study of the impact of sewage sludge. Plant Soil 226:179–188. doi:10.1023/A:1026475925703

Jacquot-Plumey E, van Tuinen D, Chatagnier O, Gianinazzi S, Gianinazzi-Pearson V (2001) 25S rDNA-based molecular monitoring of glomalean fungi in sewage sludge-treated field plots. Environ Microbiol 3:525–531. doi:10.1111/j.1462-2920.2001.00219.x

Jansa J, Smith FA, Smith SE (2008) Are there benefits of simultaneous root colonization by different arbuscular mycorrhizal fungi? New Phytol 177:779–789. doi:10.1111/j.1469-8137.2007.02294.x

Koide RT (2000) Functional complementarity in the arbuscular mycorrhizal symbiosis. New Phytol 147:233–235. doi:10.1111/j.1469-8137.2000.00710.x

Koske RE, Gemma JN (1989) A modified procedure for staining roots to detect VA mycorrhizas. Mycol Res 92:486–505. doi:10.1016/S0953-7562(89)80195-9

Munkvold L, Kjøller R, Vestberg M, Rosendahl S, Jakobsen I (2004) High functional diversity within species of arbuscular mycorrhizal fungi. New Phytol 164:357–364. doi:10.1111/j.1469-8137.2004.01169.x

Pearson JN, Abbott LK, Jasper DA (1993) Mediation of competition between 2 colonizing VA mycorrhizal fungi by the host plant. New Phytol 123:93–98. doi:10.1111/j.1469-8137.1993.tb04534.x

Pivato B, Mazurier S, Lemanceau P, Siblot S, Berta G, Mougel C, van Tuinen D (2007) *Medicago* species affect the community composition of arbuscular mycorrhizal fungi associated with roots. New Phytol 176:197–210. doi:10.1111/j.1469-8137.2007.02151.x

Püschel D, Rydlová J, Vosátka M (2007) Mycorrhiza influences plant community structure in succession on spoil banks. Basic Appl Ecol 8:510–520. doi:10.1016/j.baae.2006.09.002

Rhody D, Stommel M, Roeder C, Mann P, Franken P (2003) Differential RNA accumulation of two beta-tubulin genes in arbuscular mycorrhizal fungi. Mycorrhiza 13:137–142. doi:10.1007/s00572-002-0209-2

Seddas PMA, Arias CM, Arnould C, van Tuinen D, Godfroy O, Benhassou HA, Gouzy J, Morandi D, Dessaint F, Gianinazzi-Pearson V (2009) Symbiosis-related plant genes modulate molecular responses in an arbuscular mycorrhizal fungus during early root interactions. Mol Plant Microbe Interact 22:341–351. doi:10.1094/MPMI-22-3-0341

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic, London

Tisserant B, Gianinazzi-Pearson V, Gianinazzi S, Gollotte A (1993) In planta histochemical staining of fungal alkaline phosphatase activity for analysis of efficient arbuscular mycorrhizal infections. Mycol Res 97:245–250. doi:10.1016/S0953-7562(09)80248-7

Tisserant B, Gianinazzi S, Gianinazzi-Pearson V (1996) Relationships between lateral root order, arbuscular mycorrhiza development, and the physiological state of the symbiotic fungus in *Platanus acerifolia*. Can J Bot 74:1947–1955. doi:10.1139/b96-233

Trouvelot A, Kough JL, Gianinazzi-Pearson V (1986) Mesure du taux de mycorhization VA d'un systeme radiculaire. Recherche de methodes d'estimation ayant une signification fonctionnelle. In: Gianinazzi-Pearson V, Gianinazzi S (eds) Physiological and genetical aspects of mycorrhizae. INRA, Paris, pp 217–221

van der Heijden MGA, Boller T, Wiemken A, Sanders IR (1998) Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. Ecology 79:2082–2091. doi:10.1890/0012-9658(1998) 079[2082:DAMFSA]2.0.CO;2

van Tuinen D, Jacquot E, Zhao B, Gollotte A, Gianinazzi-Pearson V (1998a) Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol Ecol 7:879–887. doi:10.1046/j.1365-294x.1998.00410.x

van Tuinen D, Zhao B, Gianinazzi-Pearson V (1998b) PCR in studies of AM fungi: from primers to application. In: Varma AK (ed) Mycorrhiza Manual. Springer, Heidelberg, pp 387–399

Vierheilig H (2004) Further root colonization by arbuscular mycorrhizal fungi in already mycorrhizal plants is suppressed after a critical level of root colonization. J Plant Physiol 161:339–341. doi:10.1078/0176-1617-01097

Violi HA, Treseder KK, Menge JA, Wright SF, Lovatt CJ (2007) Density dependence and interspecific interactions between arbuscular mycorrhizal fungi mediated plant growth, glomalin production, and sporulation. Can J Bot 85:63–75. doi:1139/B06-151

Weidmann S, Sanchez L, Descombin J, Chatagnier O, Gianinazzi S, Gianinazzi-Pearson V (2004) Fungal elicitation of signal transduction-related plant genes precedes mycorrhiza establishment and requires the dmi3 gene in *Medicago truncatula*. Mol Plant–Microbe Interact 17:1385–1393. doi:094/MPMI.2004.17.12.1385

Wilson JM (1984) Competition for infection between vesicular arbuscular mycorrhizal fungi. New Phytol 97:427–435. doi:111/j.1469-8137.1984.tb03608.x

Wilson JM, Trinick MJ (1983) Infection development and interaction between vesicular-arbuscular mycorrhizal fungi. New Phytol 93:543–553. doi:10.1111/j.1469-8137.1983.tb02705.x

Zhu YG, Smith SE, Barrit AR, Smith FA (2001) Phosphorus (P) efficiencies and mycorrhizal responsiveness of old and modern wheat cultivars. Plant Soil 237:249–255. doi:10.1023/A:1013343811110