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ORIGINAL PAPER

Mycorrhizal status and diversity of fungal endophytes in roots of common buckwheat (*Fagopyrum esculentum*) and tartary buckwheat (*F. tataricum*)

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Abstract To determine the mycorrhizal status and to identify the fungi colonising the roots of the plants, common buckwheat (*Fagopyrum esculentum*) and tartary buckwheat (*F. tataricum*) were inoculated with an indigenous fungal mixture from a buckwheat field. Root colonisation was characterised by the hyphae and distinct microsclerotia of dark septate endophytes, with occasional arbuscules and vesicles of arbuscular mycorrhizal fungi. Sequences of arbuscular mycorrhizal fungi colonising tartary buckwheat clustered close to the *Glomus* species group A. Sequences with similarity to the *Ceratobasidium/Rhizoctonia* complex, a putative dark septate endophyte fungus, were amplified from the roots of both common and tartary buckwheat. To the best of our knowledge, this is the first report of arbuscular mycorrhizal colonisation in tartary buckwheat and the first molecular characterisation of these fungi that can colonise both of these economically important plant species.

Keywords Arbuscular mycorrhiza · Chytridiomycetes · Dark septate endophytes · Microsclerotia

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Introduction

The majority of terrestrial ecosystems are dominated by plants that form associations with mycorrhizal fungi and/or dark septate endophytes (DSE) (Smith and Read 1997), among which the arbuscular mycorrhiza (AM) are the most frequent (Smith and Read 1997; Redecker et al. 2000). Mycorrhizal fungi provide plants with mineral nutrients, especially phosphorus, in exchange for carbon compounds (Johnson et al. 2002; Bücking and Shachar-Hill 2005; Li et al. 2006), and they protect their hosts against biotic and abiotic stresses (Azcon-Aguilar and Barea 1996; Ruíz-Lozano 2003; Vogel-Mikuš et al. 2006). DSE are frequent colonisers of plant roots under extreme environmental conditions (Read and Haselwandter 1981; Barrow 2003). Despite their tremendous importance in plant growth and development, little is known about DSE identity and ecology, and their effects on the plants that they colonise (Jumpponen and Trappe 1998a). While DSE isolates have been shown to help in the uptake of nitrogen and phosphorus into plants (Haselwandter and Read 1982; Jumpponen and Trappe 1998b), their effects on overall plant biomass appear to be dependent on the host–symbiont association and on the soil nutrient status (Fernando and Currah 1996; Jumpponen and Trappe 1998b).

Common and tartary buckwheat (*Fagopyrum esculentum* and *Fagopyrum tataricum*) are important nutraceuticals because of their high content of flavonoids, minerals and vitamins, and their nutritionally balanced amino-acid composition (Rout and Chrungoo 1999; Kreft et al. 1999; Gaberščik et al. 2002; Bonafaccia et al. 2003). These crops have been shown to grow well even under poor soil conditions (Khan et al. 2005). While Virant and Kajfež-Bogataj (1988) reported mycorrhizal colonisation of common buckwheat roots, based on their characteristic features

Table 1 Reaction mixtures and PCR conditions for the ITS1F–ITS4, MH2–MH4 and NS31–AM1 primer pairs

	Primer pair		
	ITS1F–ITS4	MH2–MH4	NS31–AM1
Reaction mixture	2.5 µl 10× PCR buffer	2.5 µl 10× PCR buffer	2.5 µl 10× PCR buffer
	2.5 mM $MgCl_2$	1.5 mM $MgCl_2$	1.5 mM $MgCl_2$
	200 µM each nucleotide	200 µM of each nucleotide	200 µM of each nucleotide
	500 nM of each primer	400 nM of each primer	400 nM of each primer
	0.75 U DNA polymerase	1.25 U DNA polymerase	1.25 U DNA polymerase
	12.5 µl of template	1 µl of template	1 µl of template
PCR conditions	1 min at 94°C	2 min at 95°C	2 min at 95°C
	35 cycles:	32 cycles:	30 cycles:
	35 s at 94°C	1 min at 94°C	1 min at 94°C
	53 s at 55°C+5 s/cycle	1.5 min 48°C−0.1°C/cycle	1 min at 62°C
	30 s at 72°C	2 min at 72°C	1 min at 72°C
	10 min at 72°C	8 min at 72°C	10 min at 72°C

like hyphae, vesicles, arbuscules and intraradical spores, Harley and Harley (1987), Gai et al. (2006) and Wang and Qiu (2006) did not note any AM colonisation in common buckwheat and have therefore listed this plant as a non-mycorrhizal species. The present study reports on the identities of the fungi colonising the roots of both common and tartary buckwheat.

Materials and methods

Plant growth conditions and fungal inoculation

Seeds of the common buckwheat (*Fagopyrum esculentum* Moench, cv. Siva) and the tartary buckwheat (*F. tataricum* Gaertn.; domestic population from Luxembourg) were

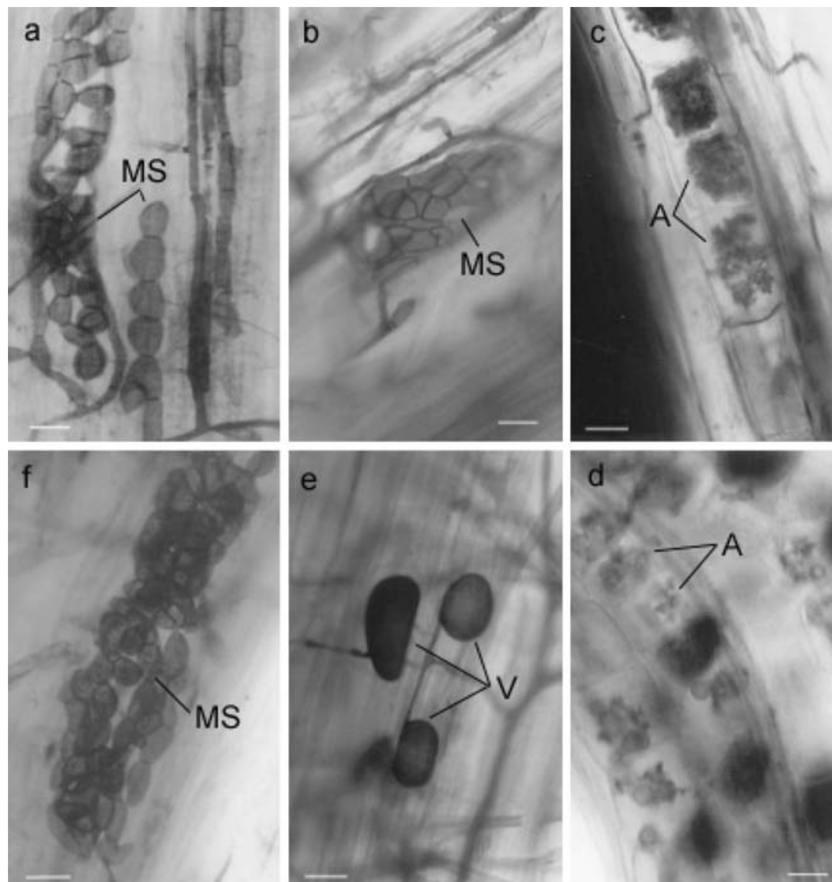


Fig. 1 Fungal structures in buckwheat roots after inoculation with the indigenous fungal mixture. Common buckwheat (**a**–**c**); tartary buckwheat (**d**–**f**). Legend: *A* arbuscules, *MS* microsclerotia, *V* vesicles. *Bars*, 25 µm

surface sterilised for 5 min with a mixture of water and sodium hypochlorite (3% active chlorine), followed by rinsing with sterile water. The seeds were sown in plastic trays (30 seeds tray^{-1}) containing a sterilised (twice at 121°C, 60 min) field soil and vermiculite mixture (1:3, *v*/*v*), with them being layered with a 1-cm depth of fungal inoculum mixture prepared from buckwheat field soil that was planted with maize (*Zea mays* L.), as the host plant. Inoculated plants were grown in an air-conditioned glasshouse at 22°C, under environmental light conditions. Plants of common and tartary buckwheat were harvested at seed maturity (84 and 91 days after germination, respectively).

Fungal structures

At harvest, fresh roots from plants belonging to both species were washed, cleared with 10% KOH and stained in 0.05% Trypan blue (Phillips and Hayman 1970). The presence of AM fungi and DSE structures was evaluated according to Trouvelot et al. (1986). Non-septate hyphae with vesicles and arbuscules were counted as AM, whereas inter/intracellular melanised hyphae with microsclerotia were recorded as DSE. The mycorrhizal frequency (F%) and the density of arbuscules in the root systems (A%) were determined. The density of microsclerotia (MS%) in the root systems were determined in the same way as the arbuscular density. For each determination of the degree of colonisation, 15 root fragments were examined per plant specimen (n=14 per species), under an Axioskop 2 MOT microscope (Carl Zeiss, Goettingen, Germany) equipped with an Axiocam MRc colour digital camera (Carl Zeiss Vision, Halbergmoos, Germany).

PCR amplification

The freshly harvested roots were washed with sterile water and frozen in liquid nitrogen prior to DNA extraction. The total genomic DNA was isolated from the root tissues according to Gardes and Bruns (1996). The ITS region of the rRNA operon was amplified using the ITS1F–ITS4

Table 2 GenBank accession numbers of the fungal sequences isolated from the common buckwheat (*Fagopyrum esculentum*) and tartary buckwheat (*F. tataricum*) roots, with the two nearest matches and corresponding E values

Fungal group	Sequence	rDNA region sequenced	Nearest match (E)		Isolated from
Ascomycota	Dothideomycetes	ITS	EF154350	DQ657853 Ascomycete sp. (0.0)	*F. esculentum*
			EF154351	EU003027 Uncultured fungus (0.0)	
			EF154352		*F. tataricum*
Basidiomycota	*Ceratobasidium*	ITS	EF154356	DQ102413 *Ceratobasidium* sp. AG-A (0.0)	*F. esculentum*
				DQ102411 *Ceratobasidium* sp. AG-A (0.0)	
		18S	EF154337	AF202282 Basidiomycete sp. (0.0)	
				D85646 *Rhizoctonia* sp. AG-A (0.0)	
		18S	EF154339	D85646 *Rhizoctonia* sp. AG-A (0.0)	
				DQ520098 *Ceratobasidium* sp. (0.0)	
	Tricholomataceae	18S	EF154340	AY916753 *Moniliophthora* sp. (0.0)	
				AY916719 *Marasmius* sp. (0.0)	
	Ceratobasidium	ITS	EF154354	AJ242903 *Rhizoctonia* sp. (0.0)	*F. tataricum*
			EF154355	DQ102413 *Ceratobasidium* sp. AG-A (0.0)	
		18S	EF154347	D85646 *Rhizoctonia* sp. AG-A (0.0)	
				EF154339 *Ceratobasidium* sp. (0.0)	
		18S	EF154353	AJ242892 *Rhizoctonia* sp. SIR-2 (0.0)	
				DQ102416 *Ceratobasidium* sp. AG-A (0.0)	
	Polyporales	18S	EF154338	DQ092911 *Pterula echo* (0.0)	
			EF154344	DQ851579 *Phyllotopsis nidulans* (0.0)	
Glomeromycota	*Glomus*	18S	EF154345	AM746135 Uncultured *Glomus* (0.0)	*F. tataricum*
			EF154346	AJ563896 Uncultured *Glomus* (0.0)	
			EF154348		
		18S	EF154349	AM746135 Uncultured *Glomus* (0.0)	
				EF177547 Uncultured *Glomus* (0.0)	
Chytridiomycota	Spizellomycete	18S	EF154341	DQ322624 *Olpidium brassicae* (0.0)	*F. esculentum*
				AF164245 *Powellomyces* sp. (1e-180)	
		18S	EF154336	AF164245 *Powellomyces* sp. (0.0)	*F. esculentum*
			EF154342	M59759 *Spizellomyces acuminatus* (0.0)	
			EF154343		*F. tataricum*

Sequences with identical GenBank matches and host species are represented together

primer pair (White et al. 1990; Gardes and Bruns 1993) for identification of ascomycetes and basidiomycetes, while the AM fungi were identified by amplification of the 18S rDNA with the MH2–MH4 primers (Vandenkoornhuyse and Leyval 1998) followed by a second (nested) PCR with the NS31 (Simon et al. 1992) and AM1 (Helgason et al. 1998) primers. The PCR conditions and reaction mixtures are given in Table 1.

Cloning, sequencing and sequence analysis

The amplicons were cleaned and ligated with pGEMT-Easy vector (Promega, Madison, WI, USA). The recombinant vector was used for transforming cells of *Escherichia coli* JM109. The transformants were plated on LB agar plates containing 50 μg/ml ampicillin and X-Gal/IPTG. Screening for recombinant cells was carried out by blue/white selection. Prior to cycle-sequencing reactions with the T7/SP6 primers, the presence of inserts in the vectors was confirmed with colony PCR using the same primer pair.

The nucleotide sequences were subjected to BLAST analysis to determine their homology with other sequences available in the databank. The CLUSTAL package (Thompson et al. 1994) was used to align the sequences with the corresponding fungal ITS rDNA and 18S rDNA sequences, using the default option of gapped-BLAST (Altschul et al. 1997). Distance analysis of the alignment data was carried out using MEGA3 software (Kumar et al. 2004), and maximum parsimony was performed in PAUP version 4.0b10 (Swofford 2003).

Results and discussion

The microscopic examination of stained root segments of common buckwheat revealed the presence of hyphae and distinct microsclerotia of DSEs (Fig. 1a,b) as well as the occasional arbuscules (Fig. 1c) and vesicles of AM fungi. The mycorrhizal frequencies (F%) and microsclerotia densities (MS) for common buckwheat ranged from 87%

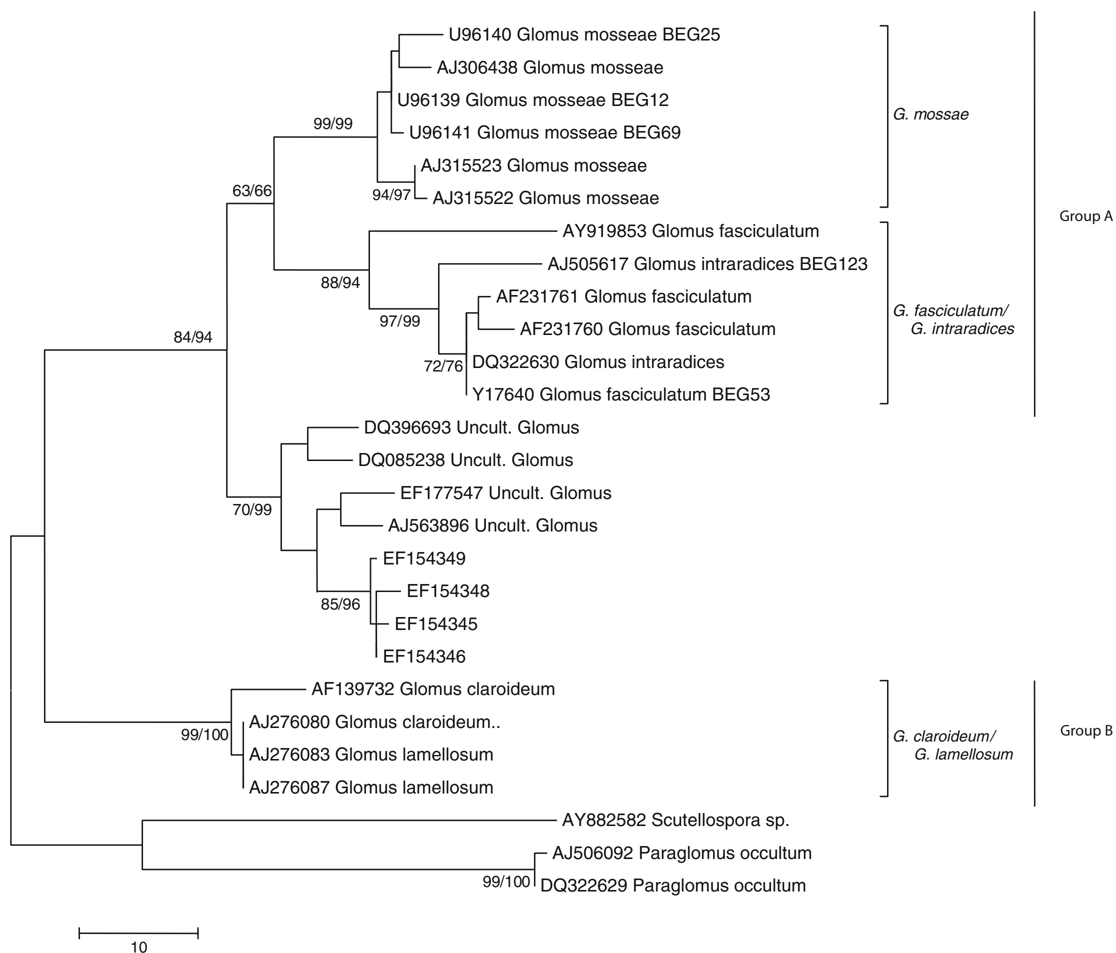


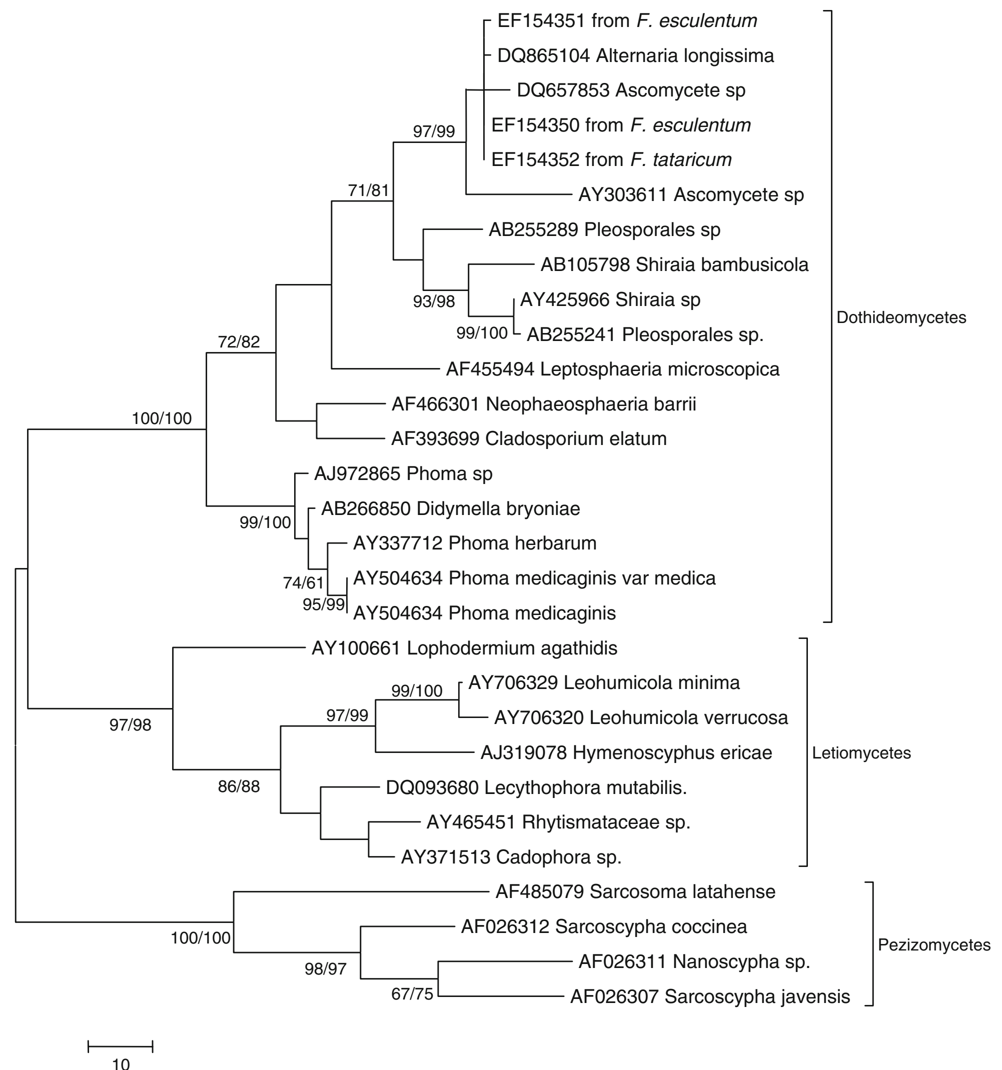
Fig. 2 Phylogenetic placement of the AM fungi associated with the roots of tartary buckwheat in one of the most parsimonious trees based on the 18S rDNA tree with topography similar to the neighbour-joining tree. *Paraglomus* and *Scutelospora* were used as an outgroup. Percentages represent bootstrap values above 60% for both maximum parsimony and NJ (MP/NJ) analysis (n=1,000 for both)

to 100% and from 0.5% to 26%, respectively. The arbuscule density (A%) was <1%. Similarly, for the roots of tartary buckwheat, DSE hyphae and microsclerotia (Fig. 1d), as well as vesicles (Fig. 1e) and rare arbuscules of AM fungi (Fig. 1f), were seen (Fig. 1d–f). Here, the mycorrhizal frequencies ranged from 87% to 100% and the microsclerotia densities from 2% to 40%. The arbuscule densities ranged from 0% to 3%. Two distinct types of microsclerotia were found in the roots of both of these buckwheat species. The first, predominant, type was composed of more relaxed groupings of rounded cells (Fig. 1a,d), whereas in the second type, hyphae that formed microsclerotia were more densely packed (Fig. 1b).

In the present study, fungal structures of both AM fungi (rare arbuscules, hyphae and vesicles) and DSEs (hyphae and microsclerotia) were seen for both common and tartary buckwheat inoculated with the indigenous fungal mixture. To our knowledge, this is the first report of the colonisation of tartary buckwheat with AM fungi and the first report of the colonisation of both buckwheat species by DSE fungi. Previously, Virant and Kajfež-Bogataj (1988) already reported mycorrhizal colonisation of common buckwheat roots characterised by hyphae, vesicles, arbuscules and intraradical spores under controlled conditions, whereas no AM structures were seen on the roots of the wild-grown common buckwheat in grassland ecosystems (Gai et al. 2006). The discrepancy in mycorrhizal status of buckwheat species in different studies could be a result of very low colonisation levels with AM fungi, as observed also in our study.

Analysis of the amplified 18S and ITS rDNA fungal sequences from the roots of both buckwheat species revealed a high level of similarity with nucleotide sequences from species belonging to Ascomycota, Basidiomycota, Glomeromycota and Chytridiomycota. The sequence data have been submitted to the GenBank database under the accession numbers EF154336 to EF154356 (Table 2). Four sequences were similar to species belonging to the *Glomus*

Fig. 3 Phylogenetic placement of the ascomycetes associated with the roots of tartary buckwheat in one of the most parsimonious trees based on the ITS rDNA tree with topography similar to the neighbour-joining tree. Percentages represent bootstrap values above 60% for both maximum parsimony and NJ (MP/NJ) analysis (n=1,000 for both)



group (Table 2). In addition, both species of buckwheat appeared to be colonised by the same dothideomycetous fungus, since the sequences EF154350 and EF154352 showed 100% similarity (Table 2). Some of the 18S rDNA amplified fungal sequences grouped within the *Ceratobasidium/Rhizoctonia* complex, with two sequences showing close resemblance to *Pterula* and *Phyllotopsis*, from Polyporales, and one sequence showing similarity to *Marasmius* and *Moniliophtora* (Tricholomataceae).

The dataset used to reconstruct the 18S maximum parsimony tree of the AM fungal sequences associated with roots of tartary buckwheat (Fig. 2) contained 518 characters, of which 362 were constant, 62 parsimony uninformative and 94 parsimony informative. The heuristic search recovered 133 equally most parsimonious trees with a length of 229 steps, a CI of 0.70, an RI of 0.84 and a rescaled CI (RCI) of 0.58.

The dataset used to reconstruct the ITS maximum parsimony tree of ascomycetes associated with the roots of tartary buckwheat (Fig. 3) contained 565 characters, of which 196 were constant, 72 parsimony uninformative and 297 parsimony informative. The heuristic search recovered 27 most parsimonious trees with a length of 509 steps, a CI of 0.56, an RI of 0.77 and an RCI of 0.43.

The phylogenetic tree constructed with the maximum parsimony method showed the sequences from AM fungi in association with tartary buckwheat as a separate clade, composed of uncultured *Glomus* species and positioned close to members of the *Glomus* group A (Fig. 2). Here, the amplification of nucleotide sequences indicates the colonisation of the roots of tartary buckwheat by AM fungi. The absence of any AM sequences in the DNA amplified from the roots of common buckwheat was attributed to the very low colonisation levels, which may have led to non-specific amplification of the AM1–NS31 primer pair in the absence of the target fungal DNA (Helgason et al. 2002; Douhan et al. 2005) and the fact that morphology does not provide a very clear-cut distinction between different mycorrhizal groups, thus leading to the possibility that non-mycorrhizal fungi are a lot more abundant in the buckwheat roots than the mycorrhizal fungi.

DSE colonisation of both buckwheat species was characterised by septate melanised hyphae and distinct microsclerotia. Sequencing of the fungal 18S rDNA revealed the presence of several species from the phylum Basidiomycota. The majority of the sequences belonged to putative DSE fungi with similarities to the *Ceratobasidium/Rhizoctonia* complex (anastomosis group A). Many *Ceratobasidium* species have a *Rhizoctonia* anamorph, which is a parasite on herbaceous plants, including the genus *Fagopyrum* (Morrall and McKenzie 1975), although they are also known as DSE colonisers of various herbaceous and woody plants (Jumpponen and Trappe 1998a). Sequencing of the fungal ITS rDNA also confirmed the presence of the *Ceratobasidium/Rhizoctonia* complex and revealed the presence of an unknown dothideomycete positioned close to Pleosporales (Fig. 3), which also colonised both of these buckwheat species. In contrast, sequences belonging to the fungal group Polyporales were only amplified from roots of tartary buckwheat, while members of Tricholomataceae were only seen on the roots of common buckwheat. The species-specific appearance of these sequences is probably a result of the marginal colonisation of buckwheat roots by these fungi.

These results clearly demonstrate that common and tartary buckwheat are penetrated by AM fungi. This is the first report of AM colonisation in tartary buckwheat. Furthermore, we have also shown colonisation of both of these buckwheat species with DSE fungi. Molecular characterisation of the colonising fungi has revealed that AM fungi are represented by relatives of a group A *Glomus* species. In addition, several sequences from the *Ceratobasidium/Rhizoctonia* complex, putative DSE fungi, were amplified from the roots of both of these buckwheat species. Despite the observed colonisation of buckwheat roots, further work is required to understand the potential roles of these fungi in the growth and development of both of these buckwheat species.

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