Diversity of symbiotic root endophytes of the *Helotiales* in ericaceous plants and the grass, *Deschampsia flexuosa*

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Abstract: Root endophyte fungi of ericaceous plants were compared with those obtained from the dominant grass in Dutch heathlands, *Deschampsia flexuosa*. We investigated the phylogenetic relatedness of these fungi and their effects on nutrient uptake in both *Calluna vulgaris* and *D. flexuosa* seedlings in synthesis trials *in vitro*. Molecular analysis based on nuclear ribosomal internal transcribed spacer (ITS) region sequences revealed that four grass root endophytes belonged to the *Helotiales* (*Ascomycetes*). The majority of the ericaceous root isolates (68 %) also clustered within the *Helotiales* and showed a remarkably high diversity. Other important fungal groups included *Phialocephala fortinii*-like fungi, making up 22 % of isolates, and *Cryptosporiopsis* species, making up 8 %. Results of the synthesis trials showed that both grass root and ericaceous isolates colonized roots of both test host species successfully and could be seen to significantly enhance nitrogen uptake of inoculated *D. flexuosa* and *C. vulgaris* seedlings when these were compared to the uninoculated controls. We conclude that beneficial, helotialean fungi associate with roots of *D. flexuosa* and that these form a group potentially overlapping in phylogeny and function with endophytes from *Ericaceae*.

Key words: *Calluna vulgaris*, *Deschampsia flexuosa*, diversity, *Helotiales*, nitrogen uptake, root endophytes, synthesis trials.

INTRODUCTION

In the Netherlands, *Deschampsia flexuosa* has become a dominant grass species in heathlands due to high deposition rates of atmospheric nitrogen, derived substantially from industrial and agricultural sources and amounting to as much as 45 kg N ha/yr (Berg & Verhoef 1998, van Oene *et al*. 1999). The competitive success of this grass is thought to be due to a growth rate that is higher than that of ericaceous shrubs (Berendse & Elberse 1990, Berendse 1998). Recently it has been shown that this grass is also able to absorb considerable amounts of organic nitrogen. This enables it to use nitrogen forms other than ammonium and nitrate, thus reducing its dependence on nitrogen mineralization in the soil (Näsholm *et al*. 1998, Falkengren-Grerup *et al*. 2000, Persson *et al*. 2003). Although the type of mycorrhizal association seen in *D. flexuosa* has received some attention, the role of mutualistic endophytic fungi in organic nitrogen uptake by this species remains uncertain (Persson & Näsholm 2001, Persson *et al*. 2003).

Deschampsia flexuosa can be colonized by multiple types of mutualistic fungi. The most common colonizers are arbuscular mycorrhizal fungi (AMF) in the *Glomeromycota*. Colonisation is seen in the production of vesicles and arbuscules in root epidermal cells (Harley & Harley 1987, Smith & Read 1997). *Deschampsia flexuosa* in alpine plant communities is also colonized by fungi with dark septate hyphae, and these fungi produce microsclerotia within and between epidermal cells (Read & Haselwandter 1981). Similar fungi frequently belong to a group known as dark septate endophytes (DSE), which are *Ascomycota*. Some members of this group appear to be mycorrhizal, at least in some hosts and habitats, including *Phialophora finlandica* Wang & Wilcox and *Phialocephala fortinii* Wang & Wilcox (Smith & Read 1997, Jumpponen 2001). *Phialocephala fortinii* is the most studied representative of the DSE complex, and seems to be distributed throughout the temperate Northern Hemisphere without showing apparent host specificity (Jumpponen & Trappe 1998, Ahlich *et al*. 1998, Addy *et al*. 2000, Grünig *et al*. 2002a). The mutualistic status of *P. fortinii* is debated, because no nutrient-exchange interfaces comparable to those of mycorrhizas have been identified (Jumpponen & Trappe 1998). Reports on the effects of *P. fortinii* on host plants reveal relationships that seem to range from parasitism to mutualism (Jumpponen & Trappe 1998, Jumpponen 2001). However, these differences can possibly be attributed to the use of undefined isolates and to experimental designs that favour either *P. fortinii* or the host (Addy *et al*. 2000).

Read and Haselwandter (1981) estimated colonisation levels of AMF and dark septate hyphae in *D. flexuosa* roots collected from an Austrian alpine ecosystem. In *D. flexuosa* they found that AMF colonisation was on average 40 % and the amount of dark septate hyphal colonisation was estimated at between 1–10 %. The identity of the dark septate fungal partner in the *D. flexuosa* roots remained unresolved. Vrålstad *et al*. (2002a) suggested that some *D. flexuosa* endophytes could belong to the *Helotiales* (*Ascomycota*). The *Helotiales* is a diverse fungal order in which the ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf & Kornan (see note on this name, next paragraph) is classified along with the DSE species *P. fortinii* and *P. finlandica*. In a recent phylogenetic analysis, the *H. ericae* aggregate also appeared to include a group of closely related, more or less darkly pigmented root-associated ascomycetes (Vrålstad *et al*. 2002a). Further evaluation is needed, however, because the analysis in question used the *Rhytismatales* as an outgroup. Gernandt *et al*. (2001) showed with small subunit nuclear ribosomal DNA sequences that *Rhytismatales* and *Helotiales* belong to the same order. The affinities of the grass root endophytes are best tested with a different outgroup.

After this manuscript was written, we became aware of the reclassification of *H. ericae* as *Rhizoscyphus ericae* Zhuang & Korf (Zhang & Zhang 2004). We follow the reasons outlined by Hambleton and Sigler (2005–this volume) for changing our usage to this new correct name, while still referring to broad group of isolates related to this species as the *H. ericae* aggregate, and retaining the designation "*Hymenoscyphus* sp." for sequences downloaded from GenBank that are connected to isolates in the *H. ericae* aggregate but not identified at the species level.

The role of AMF in organic nitrogen uptake is negligible. Arbuscular mycorrhizal fungi (AMF) mainly promote plant growth by enhancing uptake of inorganic phosphate. They are not able to capture nutrients from organic nitrogen sources, e.g. glycine (Smith & Read 1997, Hodge 2001). In contrast, ericoid mycorrhizal (ERM) fungi facilitate the uptake of organic nitrogen. This is due to their saprotrophic abilities. Proteins and amino acids are released from protein polyphenol complexes by the activity of a range of hydrolytic and oxidative enzymes (Cairney & Burke 1998, Bending & Read 1996, 1997). For example, Sokolovski *et al*. (2002) showed that *Calluna vulgaris* (L.) Hull root cells increased amino acid uptake when they were mycorrhizally associated with *R. ericae*. Among the DSE species, *P. fortinii* and *P. finlandica* also have the ability to hydrolyse organic nitrogen sources such as proteins, but their precise role in organic nitrogen uptake is not clear (Jumpponen *et al*. 1998, Caldwell *et al*. 2000).

The ericoid mycorrhizal association is described as a symbiosis between mutualistic, root-endophytic ascomycetous fungi and ericaceous plant roots. Ericoid mycorrhizal (ERM) fungi form characteristic hyphal coils in epidermal root cells (Smith & Read 1997). The strains of the *H. ericae–Scytalidium vaccinii* complex (*Helotiales*) and *Oidiodendron maius* Barron [uncertain ordinal classification, formerly *Onygenales* (Guarro & Cano 2002)] are the most widely dispersed and investigated ERM fungi (Read 1996, Straker 1996, Smith & Read 1997). Recently, molecular identification showed that the diversity of ERM fungi is much larger than was once assumed (Monreal *et al*. 1999, Perotto *et al*. 2002, Vrålstad 2002a, Allen *et al*. 2003, Bergero *et al*. 2003). In addition, the host range of ERM fungi appears to include some non-ericaceous plants (Duckett & Read 1995, Bergero *et al*. 2000, Vrålstad *et al*. 2002b).

Our objective was to better understand the prevalence and function in *D. flexuosa* both of ERM fungi and of the poorly understood group of endophytes related to *R. ericae*. Therefore we investigated the phylogenetic relatedness of these fungi and their effects on nutrient uptake on both *Calluna vulgaris* and *D. flexuosa* seedlings in synthesis trials *in vitro*.

MATERIALS AND METHODS

Root collection and isolation of fungi

Deschampsia flexuosa plants with intact roots were collected in spring 2003 from heathland, forest and grass monoculture ecosystems in the central region of the Netherlands (Table 1). For each ecosystem we selected two locations, each consisting of five replicate sites. Five plants were collected at each site for a total of 30 plants. Root systems from individual healthy plants (without dark coloured, necrotic tissue) were cleaned to remove organic material as well as adhering ericaceous roots and other heterogeneous materials. Root tips were excised and surface-sterilized for 15 s with 4 % hypochlorite, followed by 30 s exposure to 70 % ethanol solution and three rinses in sterile water. Three sterilized root tips (1 cm) were placed in each Petri dish on malt extract agar [MEA; (Oxoid, Hampshire, U.K.) agar, 20 g; distilled water, 1000 mL] amended with 30 mg/L streptomycin sulphate. Plates were incubated at 20 °C and observed daily for hyphal emergence. Mycelia growing out of the root tips were transferred after about 7 d to 2 % MEA. Pure cultures were checked weekly for sporulation and the slow growing, nonsporulating isolates were divided into three different morphological groups. Cultures were roughly grouped based on colour and appearance. Morphotype 1 consisted of cultures with black colonies with white margins; morphotype 2 contained beige,

velvety isolates, and morphotype 3 contained isolates with salmon colored colonies. Above and beyond the characters mentioned, all isolates assigned to the same morphotype were highly similar to one another. Five isolates from different morphological groups and habitats were used for molecular analyses.

The ericoid endophytic fungal isolates were collected in September 2000, from roots of whole plants of *C. vulgaris* (50 plants), *Erica tetralix* (35 plants), *Empetrum nigrum* (10 plants), *Vaccinium myrtillus* (10 plants) and *V. vitis-idaea* (15 plants) from three different heather locations and adjacent forests in The Netherlands. Localities were Dwingeloo, Dwingelderveld National Park; Hoog Buurlo, Hoog Buurlosche heath and Otterlo, De Hoge Veluwe National Park. *Ericaceae* in De Hoge Veluwe National Park could not be collected at precisely the same sites where *D. flexuosa* was collected. Ericaceous endophytic fungi were isolated using the same method as was used for endophytic fungi in *Deschampsia flexuosa*. Rapidly growing, heavily sporulating mould isolates such as *Penicillium* spp., *Verticillium* spp. *Fusarium* spp., and *Trichoderma* spp., were discarded, because our intent was to focus on isolates resembling ERM fungi.

Morphological identification

Cultures were roughly grouped based on colour and appearance. Some cultures sporulating as *Cryptosporiopsis* and related species could be identified in morphological or molecular analyses. Some DSE started to sporulate after nine months storage at 4 ºC and could be identified morphologically as *P. fortinii*. Our interest was focused on ERM fungi; therefore we performed no further molecular analyses on DSE from ericoid sources, though three *P. fortinii*-like isolates, one somewhat atypical with subglobose-square conidia, were deposited in the Centraalbureau voor Schimmelcultures (CBS; Utrecht, the Netherlands) as CBS 110240–110242.

DNA extraction and sequencing

Of the 154 slow-growing cultures from the ericaceous roots, which made up 68 % of the total isolates, 40 isolates were randomly selected for phylogenetic analysis. Five isolates from *D. flexuosa* were analysed in comparison. Genomic DNA was extracted from young mycelium with the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Subsequently, PCR and restriction fragment length polymorphism (RFLP) were applied (Gardes & Bruns 1996). For approximate species-level clustering and identification, sequences were made of the internal transcribed spacer (ITS) region of the nuclear rRNA repeat using the fungus-specific primer pair ITS1-F and ITS-4 (White *et al*. 1990, Gardes & Bruns 1996, Bidartondo *et al*. 2001). The fungal ITS-RFLP patterns were produced using the restriction enzymes *Alu* I, *Hinf* I and *Mbo* I (MBI-Fermentas, Brunschwig chemie BV, Amsterdam, the Netherlands). RFLP band sizes were estimated to within an error margin of no more than 10 %. Cloned sequences were compared to the sequences in GenBank using a Blast algorithm at the NCBI homepage, http://www.ncbi.nlm.nih.gov/.

Phylogenetic analyses

Fasta searches in the combined EMBL/GenBank/DDBJ database were used to find ITS sequences similar to those we obtained (Table 2). Sequences were aligned in BioEdit (v.5.0.9, Hall 1999) and adjusted manually after the use of the automatic ClustalW option. We used a distance matrix method and prepared a neighbour-joining tree in ClustalX. Positions with gaps were excluded. Stability of clades was tested with 1000 bootstrap iterations. GenBank accession numbers of the isolates studied are given in Table 3. The *Helotiales* ingroup data set was compiled with sequences of *Helotiaceae*, *Dermateaceae* and some related root derived fungal endophytes, such as *Cryptosporiopsis rhizophila* Verkley & Zijlstra (Verkley *et al*. 2003). The onygenalean taxa *Ajellomyces capsulatus* (Kwon-Chung) McGinnis & Katz and *Coccidioides posadasii* Fisher, Koenig, White & Taylor were used as outgroup.

Quantification of fungal colonisation in roots of *D. flexuosa*

Deschampsia flexuosa roots were cleaned and then stained to determine the amount of AMF and DSE-like colonization. Roots were cleared by heating to 90 ºC in 10 % KOH (wt/vol) for three min and then rinsed with tap water. Cleared roots were boiled for two min in a 0.5 % ink-vinegar solution with pure white household vinegar (Vierheilig *et al*. 1998) and then destained in tap water with a few drops of vinegar to remove coloration from empty cells. For quantification of colonization, 60 1.0-cm root sections of each treatment were mounted on slides (four per slide); the degree of root tissue colonization was estimated by measuring the percentage of total root length colonized by a given fungal type for five plants per treatment. Root sections were examined at 400× under the compound microscope. Structures characteristic of *P. fortinii*-like fungi were noted. AMF colonization was recognized by the presence of vesicles and arbuscules connected to broad, aseptate hyphae. Both arbuscules and vesicles were typically found in the same root specimens.

Synthesis trials

In order to test the effect of the isolated grass endophytes on the nitrogen uptake of both *D. flexuosa* and *C. vulgaris* axenic seedlings, we performed synthesis

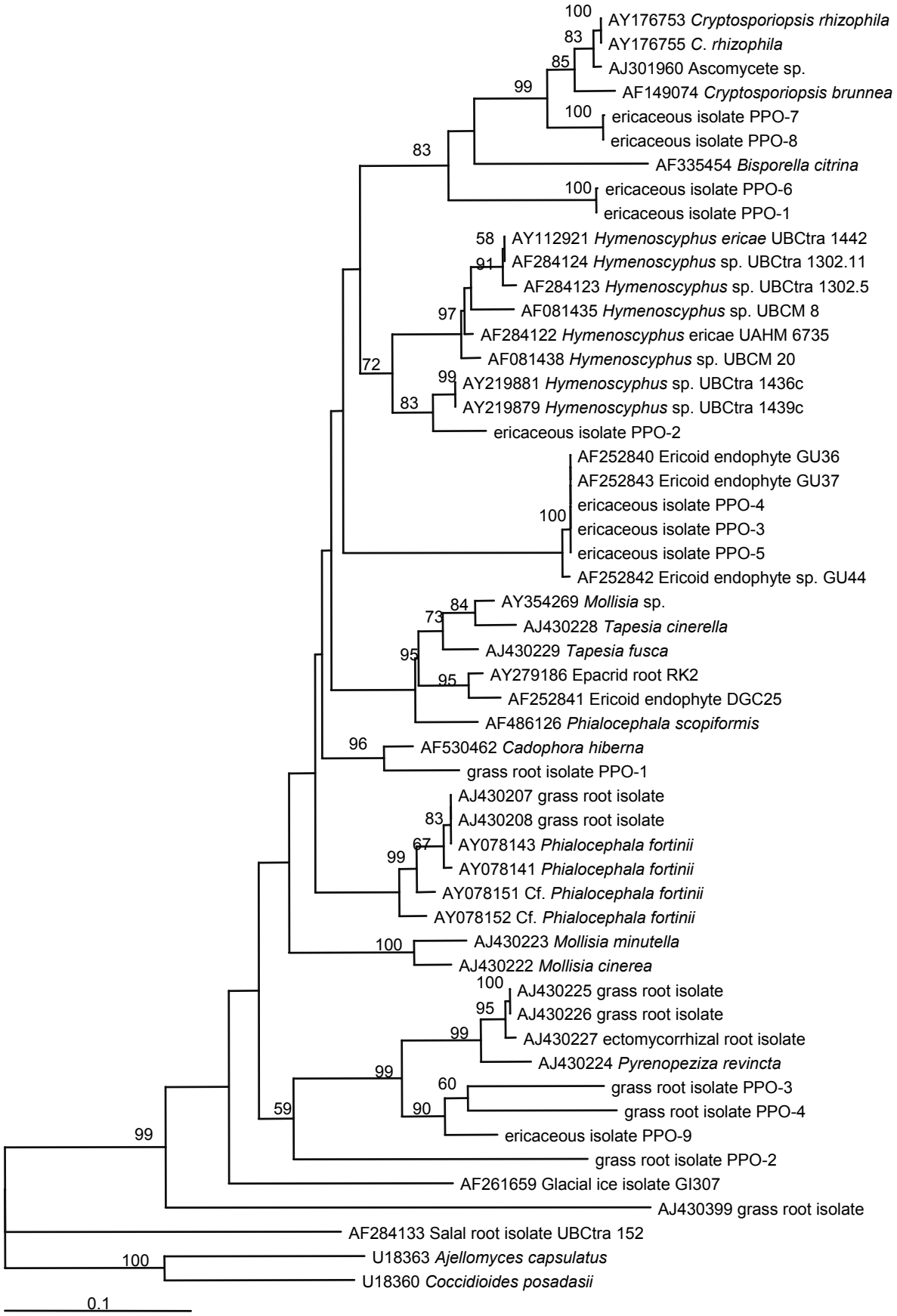

Fig. 1. Neighbour-joining tree derived from nuclear ribosomal internal transcribed spacer (ITS) sequences of endophytic root isolates from ericaceous plants (*Calluna vulgaris*, *Erica tetralix*, *Empetrum nigrum*, *Vaccinium myrtillus*, *V. vitis-idaea*) and from the grass *Deschampsia flexuosa* sampled at different locations in the Netherlands. Two species from the order *Onygenales*, *Ajellomyces capsulatus* and *Coccidioides posadasii*, were chosen as outgroup. *Hymenoscyphus ericae* aggregate sequences from GenBank are annotated with the names given in that database; for comment about current nomenclature, see main text. Bootstrap values of ≥ 50 % are indicated above the branches.

Table 1. Overview of collection sites of *Deschampsia flexuosa* in The Netherlands: ecosystem classification and dominant plant species.

Nr.	Ecosystem	Location	Dominant species
1.	Heathland	Otterlo, Nat. Park De Hoge Veluwe	*Calluna vulgaris*
			Erica tetralix
			Molinia caerulea
			Deschampsia flexuosa
2.	Heathland	Bennekom, Gemeentebosch	*Vaccinium myrtillus*
			D. flexuosa
3.	Forest	Otterlo, Nat. Park De Hoge Veluwe	*Pinus sylvestris*
			D. flexuosa
4.	Forest	Ede, Hoekelum	*P. sylvestris*
			D. flexuosa
5.	Grass monoculture	Otterlo, Nat. Park De Hoge Veluwe	*D. flexuosa*
6.	Grass monoculture	Bennekom, Gemeentebosch	*D. flexuosa*

trials with three grass endophyte isolates strongly differing in genotype and selected as representatives of three different major morphotypes obtained from *D. flexuosa*. The isolate designated "grass isolate PPO-G1" (CBS 115904) represented morphotype 1, while isolates "grass isolate PPO-G2" (CBS 115905) and "grass isolate PPO-G3" (CBS 116049) represented morphotypes 2 and 3, respectively ["PPO" designates Praktijkonderzoek Plant & Omgeving (Applied Plant Research), Wageningen University]. To compare the nutrient effect of the endophytic fungi from *D. flexuosa* with that previously described for ERM fungi, we also included three endophytic isolates from ericaceous plants, representing the most abundantly present ericaceous endophytes. These isolates included PPO-E6 (CBS 115910), a nonsporulating mycelium belonging to a species that is unnamed or not identifiable in culture, as well as the *P. fortinii* and *C. rhizophila* isolates listed in Table 4. Axenic *D. flexuosa* and *C. vulgaris* seedlings were obtained from surface sterilized seeds on water agar. Seeds were sterilized using a 5 min exposure to 1 % hypochlorite followed by three rinses in sterile water. Peat (Fixet Retailgroup, Apeldoorn, The Netherlands) with pH 4, was sieved (1.0 mm mesh size) and autoclaved twice (20 min at 120 °C). Water agar [WA; (Oxoid, Agar technical no.3, Hampshire, UK) agar, 15 g; distilled water, 1000 mL] was mixed with the sterile peat at 0.5 g peat per 9 cm Petri dish. The next day, the agar in each of these plates was cut in half and one half was removed. At the midpoint of the cut edge of the remaining half, a sterile 2-wk-old *D. flexuosa* or a 4-wk-old *C. vulgaris* seedling was placed.

Each plate was inoculated with one agar plug from an actively growing margin of a fungal colony on Modified Melin Norkrans medium [MMN; malt extract agar (Oxoid, Hampshire, U.K.), 3 g; agar (Oxoid, Agar technical no.3, Hampshire, U.K.), 15 g; D-glucose, 10 g; $(NH_4)_2HPO_4$, 0.25 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.15 g; $CaCl_2 \cdot 2H_2O$, 67 mg; $FeCl_3$ (1 % solution), 1.2 mL; NaCl, 25 mg; thiamine·HCl, 0.1 mg; distilled water, 1000 mL] (Marx 1969). Five replicates were used for each tested isolate. In the plates with the control plants, an agar plug with sterilized MMN agar was added. The plates with the inoculated and control seedlings were taped with Parafilm (Pechinay Plastic Packaging, Neenah, WI, U.S.A.) to prevent drying out and to avoid contamination. Plates were placed upright, according to a randomized block design in a growth chamber at 25 °C day temperature and 15 °C night temperature, in a 16 h / 8 h day/night cycle. They were harvested after 5 wk, when the tallest seedlings were grown up to 6 cm length.

Seedling roots were stained in a 0.2 % solution of trypan blue in lactic acid:glycerol:water (3¼ : 3 : 4 by vol.) and then transferred to a storage solution of lactic acid:glycerol:water (1 : 2 : 1 by vol.). They were mounted on a microscopic slide and examined at 400× under the compound microscope for the presence of associated hyphae and intracellular colonisation.

The shoots were dried at 70 °C for 24 h and their weight was measured with a microbalance. Dried shoots were pulverised and their N concentrations were measured using an elemental analyser (EA 1108, Fisons Instruments, Nottingham, U.K.). To obtain a value for the absolute amount of nitrogen in the shoots we multiplied the nitrogen concentration in shoots of *D. flexuosa* or *C. vulgaris* by the dry weight of the corresponding shoots at the end of the experimental period.

Table 2. Included reference material and sequences of ascomycetes of *Helotiales* (ingroup taxa) and *Onygenales* (outgroup taxa).

GenBank accession no.	Taxon	Strain	Family or Order	Host	Geographic origin	Source
Ingroup taxa of *Helotiales*:						
AJ301960	Ascomycete sp.	BBA 71218	Unclassified	*Erica* sp.	Germany	Nirenberg *et al.* (2002)
AJ430227	Axenic ectomycorrhizal root isolate	ARON 3026	Helotiaceae	*Betula pubescens*	Norway	Vrålstad *et al.* (2002a)
AJ430207	Axenic grass root isolate	ARON 2927	anamorphic *Helotiales*	*Deschampsia flexuosa*	Norway	Vrålstad *et al.* (2002a)
AJ430208		ARON 2929	anamorphic *Helotiales*	*D. flexuosa*	Norway	Vrålstad *et al.* (2002a)
AJ430399		ARON 2959	unclassified *Helotiales*	*D. flexuosa*	Norway	Vrålstad *et al.* (2002a)
AJ430225		ARON 2889	*Dermateaceae*	*Festuca ovina*	Norway	Vrålstad *et al.* (2002a)
AJ430226		ARON 2892	*Dermateaceae*	*F. ovina*	Norway	Vrålstad *et al.* (2002a)
AF335454	*Bisporella citrina*	'F 140146 (UBC)'	*Helotiaceae*	*Unknown*	Unknown	Berbee *et al.* (unpubl.)
AF530462	*Cadophora hiberna*	GB5530	anamorphic *Helotiales*	*Robinia pseudoacacia*	Spain	Bills (unpubl.)
AY176753	*Cryptosporiopsis rhizophila*	CBS 109839	anamorphic *Dermateaceae*	*E. tetralix*	The Netherlands	Verkley *et al.* (2003)
AY176755	*C. rhizophila*	CBS 110603	anamorphic *Dermateaceae*	*Calluna vulgaris*	The Netherlands	Verkley *et al.* (2003)
AY279186	Epacrid root endophyte sp.	RK 2	unknown	*Epacris microphylla*	Australia	Williams *et al.* (unpubl.)
AF252840	Ericoid endophyte sp.	GU36	unclassified *Helotiales*	*C. vulgaris*	UK	Sharples *et al.* (2000)
AF252843		GU37	unclassified *Helotiales*	*C. vulgaris*	UK	Sharples *et al.* (2000)
AF252842		GU44	unclassified *Helotiales*	*C. vulgaris*	UK	Sharples *et al.* (2000)
AF252841		DGC25	unclassified *Helotiales*	*C. vulgaris*	UK	Sharples *et al.* (2000)
AF261659	Glacial ice euascomycete	GI307	unknown	Isolated from glacial ice	Greenland	Ma *et al.* (2000)
AF284122	*Rhizoscyphus ericae*	UAMH 6735	*Helotiaceae*	*Gaultheria shallon*	Canada	Allen *et al.* (unpubl.)
AY112921	*R. ericae*	UBCtraSeq1442.1	*Helotiaceae*	*G. shallon*	Canada	Allen *et al.* (unpubl.)
AF081435	*Hymenoscyphus* sp.	UBCM8	*Helotiaceae*	*G. shallon*	Canada	Monreal *et al.* (1999)
AF081438		UBCM20	*Helotiaceae*	*G. shallon*	Canada	Monreal *et al.* (1999)
AF284123		UBCtra1302.5	*Helotiaceae*	*G. shallon*	Canada	Allen *et al.* (unpubl.)
AF284124		UBCtra1302.11	*Helotiaceae*	*G. shallon*	Canada	Allen *et al.* (unpubl.)
AY219881		UBCtra1436C	*Helotiaceae*	*G. shallon*	Canada	Allen *et al.* (unpubl.)
AY219879		UBCtra1439C	*Helotiaceae*	*G. shallon*	Canada	Allen *et al.* (unpubl.)
AY354269	*Mollisia* sp.	olrim132	*Dermateaceae*	*B. pendula*	Sweden	Lygis *et al.* (2004)
AJ430223	*M. minutella*	ARON 3129	*Dermateaceae*	*Epilobium angustifolium*	Norway	Vrålstad *et al.* (2002a)
AJ430222	*M. cinerea*	ARON 3139	*Dermateaceae*	*Picea abies*	Norway	Vrålstad *et al.* (2002a)

Table 2. (Continued).

GenBank accession no.	Taxon	Strain	Family or Order	Host	Geographic origin	Source
AY078151	Cf. *Phialocephala fortinii*	DSE-C	*Helotiaceae*	*Pinus sylvestris*	Germany	Grünig *et al.* (2002b)
AY078152		DSE-C	*Helotiaceae*	*P. abies*	Switzerland	Grünig *et al.* (2002b)
AY078141	*Phialocephala fortinii*	93-301	*Helotiaceae*	*Fagus sylvatica*	Switzerland	Grünig *et al.* (2002b)
AY078143		CBS 109313	*Helotiaceae*	*C. vulgaris*	Switzerland	Grünig *et al.* (2002b)
AF486126	*P. scopiformis*	CBS 468.94	*Helotiaceae*	*P. abies*	Germany	Grünig *et al.* (2002b)
AJ430224	*Pyrenopeziza revincta*	ARON 3150	*Dermateaceae*	*E. angustifolium*	Norway	Vrålstad *et al.* (2002a)
AF149074	*Cryptosporiopsis brunnea*	UBCtra 288	*anamorphic Dermateaceae*	*G. shallon*	Canada	Sigler *et al.* (2005)
AF284133		UBCtra 1522.5	unknown	*G. shallon*	Canada	Allen *et al.* (unpubl.)
AJ430228	*Tapesia cinerella*	ARON 3188	*Dermateaceae*	Decaying twig/bark	Norway	Vrålstad *et al.* (2002a)
AJ430229	*T. fusca*	ARON 3154	*Dermateaceae*	Decaying twig/bark	Norway	Vrålstad *et al.* (2002a)
Outgroup taxa of *Onygenales*:						
U18363	*Ajellomyces capsulatus*	–	*Onygenaceae*	unknown	unknown	Berbee *et al.* (1995)
U18360	*Coccidioides posadasii*	–	anamorphic *Onygenales*	unknown	unknown	Berbee *et al.* (1995)

Table 3. Origins and GenBank accession numbers of representative root isolates collected from *Deschampsia flexuosa* and ericaceous plants in The Netherlands.

GenBank accession no.	CBS accession no.	Description	Host plant	Geographic origin
AY599235	115904	grass PPO-G1	*Deschampsia flexuosa* (heathland)	Otterlo, Nat. Park "De Hoge Veluwe"
AY599236	115905	grass PPO-G2	*D. flexuosa* (grass)	Otterlo, Nat. Park "De Hoge Veluwe"
AY599237	116049	grass PPO-G3	*D. flexuosa* (forest)	Otterlo, Nat. Park "De Hoge Veluwe"
AY599238	115906	grass PPO-G4	*D. flexuosa* (grass)	Bennekom, Gemeentebosch
AY599239	115907	ericaceous PPO-E1	*Vaccinium myrtillus*	Hoog Buurlo, Hoog Buurlosche heide
AY599240*		ericaceous PPO-E2	*Calluna vulgaris*	Otterlo, Nat. Park "De Hoge Veluwe"
AY599241	115908	ericaceous PPO-E3	*C. vulgaris*	Otterlo, Nat. Park "De Hoge Veluwe"
AY599242	115909	ericaceous PPO-E4	*C. vulgaris*	Otterlo, Nat. Park "De Hoge Veluwe"
AY599243*		ericaceous PPO-E5	*C. vulgaris*	Otterlo, Nat. Park "De Hoge Veluwe"
AY599244	115910	ericaceous PPO-E6	*C. vulgaris*	Hoog Buurlo, Hoog Buurlosche heide
AY599245*		ericaceous PPO-E7	*C. vulgaris*	Dwingeloo, Nat. Park "Dwingelderveld"
AY599246	115911	ericaceous PPO-E8	*Empetrum nigrum*	Dwingeloo, Nat. Park "Dwingelderveld"
AY599247	115912	ericaceous PPO-E9	*C. vulgaris*	Dwingeloo, Nat. Park "Dwingelderveld"

* isolates indicated no longer viable at time of this writing.

Table 4. Fungi used to inoculate roots of *Deschampsia flexuosa* and *Calluna vulgaris* in the *in vitro* synthesis trials, and plant species and location from which they were isolated.

Endophytic species	Host plant	Geographic origin
grass PPO-G1 (Morphotype 1)	*Deschampsia flexuosa* (heathland)	Otterlo, Nat. Park De Hoge Veluwe
grass PPO-G2 (Morphotype 2)	*D. flexuosa* (grass monoculture)	Otterlo, Nat. Park De Hoge Veluwe
grass PPO-G3 (Morphotype 3)	*D. flexuosa* (forest)	Otterlo, Nat. Park De Hoge Veluwe
ericaceous isolate PPO-E6	*Erica tetralix*	Otterlo, Nat. Park De Hoge Veluwe
Cryptosporiopsis rhizophila CBS 109839	*E. tetralix*	Dwingeloo, Nat. Park Dwingelderveld
Phialocephala fortinii CBS 110241	*Vaccinium vitis-idaea*	Dwingeloo, Nat. Park Dwingelderveld

Table 5. Number of isolates of three different endophyte morphotypes isolated from roots of *Deschampsia flexuosa* sampled in heathlands, forests and grass monocultures. Different letters in vertical direction, indicate significant differences in isolation proportions among habitats (Chi-square test, $P < 0.05$). Different hyphenated numbers in horizontal direction indicate significant differences in isolation proportions among fungal groups within one habitat (Chi-square test, $P < 0.05$).

Number of isolates	Morphotype 1	Morphotype 2	Morphotype 3	Total
Heathland	28^{b2}	3^{a1}	27^{2}	58
Forest	12^{a1}	15^{b1}	35^{2}	62
Grass monoculture	6^{a1}	40^{c3}	22^{2}	68
Total	62	68	59	189

Statistical analysis

Chi square testing was used to evaluate the significance of differences in isolation proportions of *D. flexuosa* endophyte morphotypes in different habitats. It was also used to evaluate differences in morphotype and species isolation frequencies among ericaceous plant species and the locations at which they were sampled. Data for fungal colonization of *D. flexuosa* roots were arcsine-transformed to obtain a normal distribution and then tested with one-way ANOVA. Average amounts of shoot nitrogen of *D. flexuosa* and *C. vulgaris* seedlings in the fungal treatments were compared with the control treatment by one-way ANOVA. The accepted significance level for all statistical tests was $P < 0.05$.

RESULTS

Abundance and occurrence of morphological fungal groups

Culturing of 450 root tips of *D. flexuosa*, (30 plants, 15 root tips / plant) yielded 257 fungal isolates. Most were sterile mycelia. Most of these mycelia could be placed into one of three major groups based on morphological characteristics. Morphotype 1 was mostly isolated from roots of *D. flexuosa* growing in heathlands and less frequently from plants growing in grasslands or in forest devoid of ericaceous plants (Table 5). In contrast, morphotype 2 was most frequently isolated from *D. flexuosa* plants growing in grasslands. Compared to morphotype 1 and 2 isolates, morphotype 3 isolates were relatively evenly distributed across all three ecosystems.

Culturing of 414 root tips from 115 ericaceous plants yielded 227 isolates. Results for cultures that sporulated and could be characterized morphologically are shown in Tables 6, 7. The largest group consisted of *P. fortinii*-like cultures (22 %). Some cultures sporulated after being cultured for nine months at 4 ºC. Three isolates could be identified based on morphological characteristics as *P. fortinii*. The *P. fortinii*-like isolates occurred in association with most ericaceous species except *V. myrtillus*. They were isolated from all locations, but were most abundant from the Dwingeloo site. Two *Cryptosporiopsis* species made up the second most abundant group, accounting for 8 % of isolates. *C. rhizophila* occurred in most ericaceous plants except *E. nigrum*, and was only found in plants from the Hoog Buurlo and Dwingeloo sites. *Cryptosporiopsis* sp. 2 was isolated from most locations, but was not obtained from *C. vulgaris* roots. Three isolates belonging to unidentified members of the *Cryptomycetales* were obtained from two different sites at Dwingeloo. Only three isolates of *O. maius* were obtained from roots of *Vaccinium vitis-idaea*.

Table 6. List of sporulating endophytes isolated from ericaceous hosts. All strains were from roots collected in the Netherlands. Shown are CBS and GenBank accession numbers, host plant and geographic origin.

Fungal isolate **CBS accession no.** **(GenBank accession no.)**	**Nr. isol.**	**Host plant**	**Geographic origin**
*Cryptosporiopsis rhizophila**	9		
CBS 109839 (AY176753)		*Erica tetralix*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110602 (AY176754)		*Calluna vulgaris*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110603 (AY176755)		*C. vulgaris*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110604 (AY176756)		*C. vulgaris*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110606 (AY176757)		*E. tetralix*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110609 (AY176758)		*E. tetralix,*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110612 (AY176759)		*Vaccinium vitis-idaea*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110616 (AY176760)		*V. myrtillus*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110617 (AY176761)		*V. myrtillus*	Hoog Buurlo, Hoog Buurlosche heide
Cryptosporiopsis sp. 2	9		
CBS 110611		*Empetrum nigrum*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110613		*V. myrtillus*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110615		*V. myrtillus*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110614		*V. myrtillus*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110605		*E. tetralix*	Otterlo, Nat. Park De Hoge Veluwe
CBS 110608		*E. tetralix*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110652		*V. vitis-idaea*	Hoog Buurlo, Hoog Buurlosche heide
CBS 110610		*E. tetralix*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110618		*V. myrtillus*	Hoog Buurlo, Hoog Buurlosche heide
Cryptomycetales unident.	3		
CBS 110651		*E. tetralix*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110654		*E. tetralix*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110653		*V. vitis-idaea*	Dwingeloo, Nat. Park Dwingelderveld
Oidiodendron maius	3		
CBS 110450		*V. vitis-idaea*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110451		*V. vitis-idaea*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110452		*V. vitis-idaea*	Dwingeloo, Nat. Park Dwingelderveld
Phialocephala fortinii	3		
CBS 110240		*E. nigrum*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110241		*V. vitis-idaea*	Dwingeloo, Nat. Park Dwingelderveld
CBS 110242		*C. vulgaris*	Dwingeloo, Nat. Park Dwingelderveld
P. fortinii-like	46		
JA- 7, 12, 16, 45, 106, 110, 135, 138, 144, 161, 162, 164, 165, 175, 569, 350, 358, 359, 366, 367, 370, 372, 375, 507, 514, 547, 548, 550, 244, 288, 306, 331, 528, 529, 561, 400, 403, 406, 409,413, 414, 426, 430, 436, 541		*C. vulgaris* (4)	Otterlo, Nat. Park De Hoge Veluwe
		C. vulgaris (11)	Dwingeloo, Nat. Park Dwingelderveld
		E. tetralix (1)	Otterlo, Nat. Park De Hoge Veluwe
		E. tetralix (6)	Dwingeloo, Nat. Park Dwingelderveld
		E. nigrum (13)	Dwingeloo, Nat. Park Dwingelderveld
		V. vitis-idaea (1)	Hoog Buurlo, Hoog Buurlosche heide
		V. vitis-idaea (10)	Dwingeloo, Nat. Park Dwingelderveld

*See also Verkley *et al*. (2003).

Table 7. Number of isolates of five most abundant fungal morphotypes isolated from ericaceous roots sampled in three different heathland locations. Different letters within columns indicate significant differences among sites (Chi-square test, $P < 0.05$). Different hyphenated numbers within rows indicate significant differences in isolation proportions among fungal groups within one location or plant (Chi-square test, $P < 0.05$). Chi-square tests were only performed when numbers were sufficient (frequencies of expected values in contingency tables > 5).

Number of isolates	***Cryptosporiopsis rhizophila***	***Cryptosporiopsis* sp. 2**	**Dark sterile endophyte (DSE)**	**Identical to ericaceous isolate PPO-3**	**Identical to ericaceous isolate PPO-6**	**Total**
Origin:						
Total (n=110)	9^{1}	9^{1}	49^{3}	23^{2}	6^{1}	96
Otterlo, Nat. Park De Hoge Veluwe (n=35)	0	1	5^{a}	9^{b}	1	16^{a}
Hoog Buurlo, Hoog Buurlosche heide (n=25)	6	5	1^{a}	1^{a}	2	15^{a}
Dwingeloo, Nat. Park Dwingelderveld (n=50)	3^{1}	3^{1}	43^{b3}	13^{b2}	3^{1}	65^{b}
Otterlo, Nat. Park De Hoge Veluwe (n=35):						
Calluna vulgaris (n=20)	0	0	4	6	0	10
Erica tetralix (n=15)	0	1	1	3	1	6
Hoog Buurlo, Hoog Buurlosche heide (n=25):						
C. vulgaris (n=5)	2	0	0	0	1	3
E. tetralix (n=5)	1	0	0	0	0	1
Vaccinium myrtillus (n=10)	2	4	0	0	1	7
V. vitis-idaea (n=5)	1	1	1	1	0	4
Dwingeloo, Nat. Park Dwingelderveld (n=50):						
C. vulgaris (n=25)	1	0	11^{a}	4	1	17^{a}
E. tetralix (n=15)	2	2	6^{ab}	4	0	14^{ab}
Empetrum nigrum (n=10)	0	1	13^{c}	5	0	19^{c}
V. vitis-idaea (n=10)	0	0	10^{bc}	0	2	12^{abc}

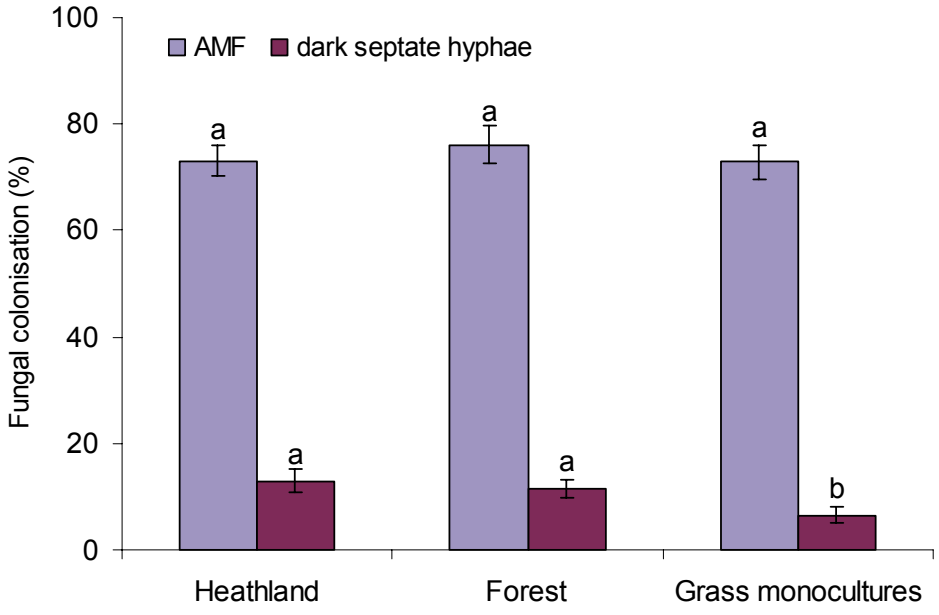

Fig. 2. Colonization levels of arbuscular mycorrhizal fungi (AMF) and dark septate hyphae in roots of *Deschampsia flexuosa* sampled in heathlands, forests and grass monocultures. Different letters indicate significant differences among habitats (one-way ANOVA, $P < 0.05$).

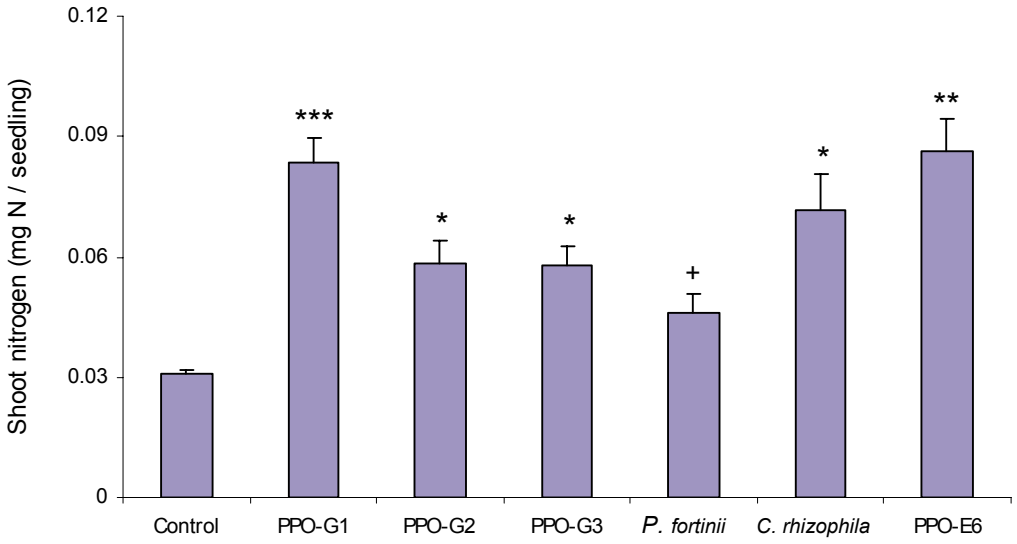

Fig. 3. Shoot nitrogen uptake in mg N of *Deschampsia flexuosa* seedlings inoculated with endophytic fungi isolated from *D. flexuosa*: PPO-G1, PPO-G2, PPO-G3 and ericoid endophytic fungal species (*Phialocephala fortinii*, *Cryptosporiopsis rhizophila* and ericaceous isolate type PPO-E6). Harvest was after 5 wk. n = 5. ANOVA: $p < 0.10^{+}$, $p < 0.05$*, $p < 0.01$**, $p < 0.001$***. Values presented are means ± 1 SE.

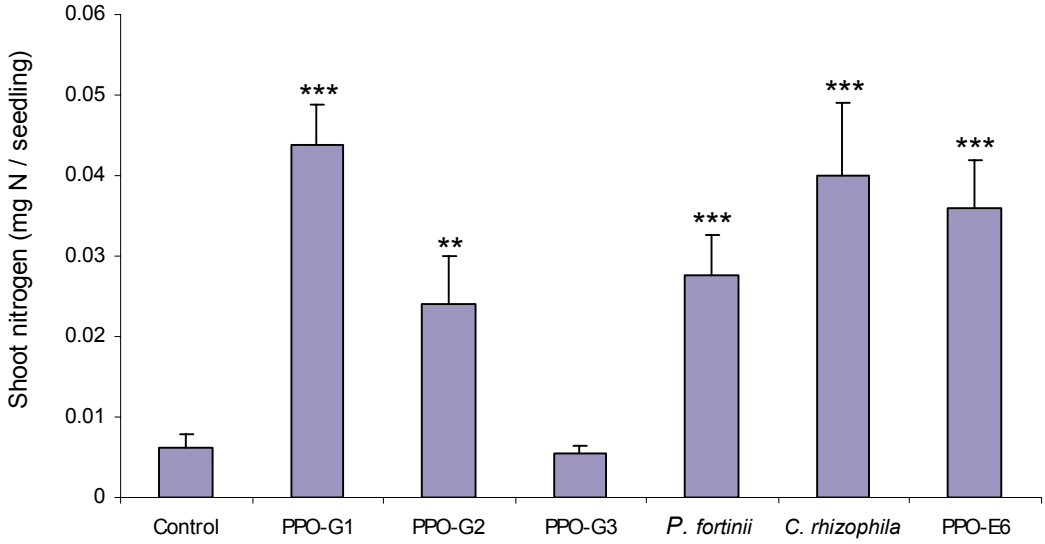

Fig. 4. Shoot nitrogen uptake in mg N of *Calluna vulgaris* seedlings inoculated with endophytic fungi isolated from *Deschampsia flexuosa*: PPO-G1, PPO-G2, PPO-G3 and ericoid endophytic fungal species (*Phialocephala fortinii*, *Cryptosporiopsis rhizophila* and ericaceous isolate type PPO-E6). Harvest was after 5 wk. n = 5. ANOVA: $p < 0.01$**, $p < 0.001$***. Values presented are means ± 1 SE.

Phylogenetic analyses

Internal transcribed spacer (ITS) sequences of five fungal isolates from grass roots were compared to sequences in GenBank. One sequence was excluded from further analysis, because it was identified as pertaining to a non-helotialean ascomycete, *Chaetosphaeria vermicularioides* (Saccardo & Roumeguère) W. Gams & Holubová-Jechová. Sequences that appeared to be helotialean and sequences of uncertain affinity were retained. Figure 1 shows the neighbour-joining tree containing the included isolates from grass roots and *Ericaceae*. Four grass isolates clustered well within the Helotiales. Grass isolate PPO-G1 (CBS 115904), which was isolated from a heathland, was closely related to *Cadophora hiberna* Bills. It was, however, unrelated to the other grass root isolates and to *Hymenoscyphus* species. The other three grass isolates formed a cluster separate from PPO-G1. Grass isolates PPO-G3 (CBS 116049) and PPO-G4 (CBS 115906) from monocultures clustered with the sequence of *Pyrenopeziza revincta* (P. Karst.) Gremmen as well as sequences of isolates from axenic roots of the grass *Festuca ovina* L. (Vrålstad *et al*. 2002a). Remarkably, the ericaceous isolate PPO-E9 (CBS 115912) clustered also in this group, with a bootstrap support of 90 %. Grass root isolate PPO-G2 (CBS 115905) from forest obtained only 59 % bootstrap support with PPO-G3 and PPO-G4.

From the 40 slow-growing ericaceous isolates selected for analysis, nine groups evidenced different RFLP patterns. Within these ericaceous isolates, isolate PPO-E2 (no longer alive) was distinct in being most similar (83 % similarity) to root-associated isolates UBCTRA1436C and UBCTRA1439C, identified as *Hymenoscyphus* sp. and isolated from the ericaceous shrub salal, *Gaultheria shallon* Pursh (Allen *et al*. 2003). A small cluster was formed by ericaceous isolates PPO-E3 (CBS 115908), PPO-E4 (CBS 115909) and PPO-E5 (no longer alive), which showed 100 % similarity with the sequences from the ericoid endophyte isolates GU36 and GU37 from *C. vulgaris* (Sharples *et al*. 2000). There was no bootstrap support for an association of this cluster with the ericoid endophyte isolate GU44, known to produce mycorrhizal coils in *Calluna* roots (Sharples *et al*. 2000). Isolates PPO-E1 (CBS 115907) and PPO-E6 (CBS 115910) formed a distinct cluster close to the *Cryptosporiopsis* species. This cluster was not closely related to the above-mentioned group of *Hymenoscyphus* isolates. Isolates PPO-E7 (no longer alive) and PPO-E8 (CBS 115911) showed 100 % mutual similarity and were supported in a clade with *C. rhizophila* and two unidentified GenBank sequences, one from *Erica* sp. and one from *G. shallon*.

Quantification of fungal colonisation in roots of *D. flexuosa*

Deschampsia flexuosa roots collected in the field contained both AMF and *P. fortinii*-like structures (Fig. 2). At all sites, AMF colonization was found at greater levels than *P. fortinii*-like colonization ($P < 0.001$). Levels with *P. fortinii*-like colonization were highest in roots from heathlands and forests; the levels in plants from monocultures were significantly lower ($P < 0.05$).

Synthesis trials

In *D. flexuosa* seedlings inoculated in synthesis trials, the amount of nitrogen in the shoots was enhanced over levels seen in controls. This was true not just when endophytic fungi from the same host were tested, but also when ericaceous isolates were used (Fig. 3). The highest amounts of nitrogen were found in seedlings colonized by grass isolate PPO-G1 and ericoid isolate PPO-E6. In all treatments, staining of inoculated grass roots showed high fungal colonization of the epidermal cells, with 80 to 93 % of cells affected. The nitrogen amount in *C. vulgaris* seedlings was increased when seedlings were inoculated with ericoid endophytic isolates from the same host. In addition, the grass isolates PPO-G1 and PPO-G4 had a positive effect on nitrogen amounts (Fig. 4). The dry weight of the seedlings showed results similar to those obtained for total shoot nitrogen levels (data not shown); however, the nitrogen concentrations of shoots were not significantly different between treatments.

DISCUSSION

In this study, we obtained a high diversity of fungal endophytes from ericaceous plants and the grass, *D. flexuosa* (Fig. 1). We obtained several species from *D. flexuosa* roots that belonged to the *Helotiales*, the taxonomic order to which the well-known mycorrhiza-formers in the genus *Hymenoscyphus* (now in part transferred to *Rhizoscyphus*) belong. Our finding that three grass root endophyte types and some ericoid endophytic fungi could enhance nitrogen uptake in *D. flexuosa* is unprecedented (Fig. 3). Still more remarkable was the finding that two grass endophyte types could increase nitrogen uptake in *C. vulgaris in vitro* (Fig. 4). To our knowledge, this is the first report that *D. flexuosa* is able to benefit when it is grown with various fungi from the *Helotiales*.

The *Helotiales*, to which four of the grass endophyte types belong, is a diverse order that includes many ericaceous endophytes (Monreal *et al*. 1999, Berch *et al*. 2002, Vrålstad *et al*. 2002a). We found no support for a close relationship of the grass root isolates with *R. ericae* or with the salal isolates identified as *Hymenoscyphus* sp. Even for grass isolate PPO-G1, representing the morphotype (morphotype 1) that was most abundantly isolated from heathland grass roots, there was no bootstrap support for a relationship with these *Hymenoscyphus* species (Fig. 1). Due to the diversity of the sequences, the tree shows long branch attraction. Nevertheless, two of our grass root isolates, PPO-G3 and PPO-G4, clearly show a close relationship with the sequences of *F. ovina* endophytes clustering in the *Dermateaceae*. Moreover, ericaceous isolate PPO-E9 falls into the same cluster, suggesting that there may be closer relationships between some grass root and ericoid endophytes than has previously been suspected.

Our work confirms that there is a large diversity of ericoid endophytes. Only a few ericaceous isolates clustered within the *H. ericae* aggregate; most were not closely related. Monreal *et al*. (1999) were also surprised by the high diversity of their ericaceous isolates, which did not seem closely related to fungi collected from ericoid roots by Xiao and Berch (1996). Isolates related to *Cryptosporiopsis* spp., making up almost 10 % of our isolates, were unusually prominent in our collection (Table 7). The ericaceous isolate types PPO-E7 and PPO-E8, though they could not be induced to sporulate, appeared to be members of this clade. *C. rhizophila* was isolated from various types of ericoid roots; as detailed above, it conferred nutrient benefits on *D. flexuosa* and *C. vulgaris* seedlings. This species, however, was not isolated from grass. Whether it is truly confined to ericaceous hosts needs to be further investigated.

With our isolation methods, we restricted ourselves primarily to ascomycetes. Recently, however, Allen *et al*. (2003) have shown that basidiomycetes such as *Sebacina* spp. are also abundantly present in salal roots. The difficulty of isolating and maintaining these fungi hampers mycorrhizal experimentation. Some sterile ascomycetous fungi are also difficult to maintain in viable condition in pure culture for several years. In the present study, representative isolates PPO-E2, PPO-E5 and PPO-E7 in our ericaceous fungal collection were lost within a short period after experiments were finished in both our participating molecular phylogeny centre and our ecological centre. The losses in our collection occurred due to bacterial infection. Sterile fungal isolates may be notably difficult to preserve in pure culture because potentially enduring conidia and spores are not formed, and resistant chlamydospores are formed only in some species (S. Tan, pers. comm.).

In this study we focussed on the ecological role of root endophytes, as occurrence in roots of a given fungus does not mean that fungus is mycorrhizal. Read (1991) emphasized the need for working according to Koch's postulates to test root-inhabiting fungi and to evaluate plant responses as seen in growth parameters or nutrient balance. Evaluation of mycorrhizal testing with ericoid endophytes is, however, mostly restricted to microscopic structural examination, generally only performed with roots obtained from synthesis trials (Monreal *et al*. 1999, Bergero *et al*. 2000, Sharples *et al*. 2000, Berch *et al*. 2002, Allen *et al*. 2003, Bergero *et al*. 2003). With mycorrhiza being defined as mutualistic symbiotic associations, bidirectional transfer of carbon and nutrients should preferably be shown to substantiate that both partners benefit in associations referred to as mycorrhizal (Smith & Read 1997, Lindahl *et al*. 2001). Such testing is planned in our research in the immediate future.

We used only a small selection of ericoid and grass root endophytes in our synthesis trials and did not test a range of nutritional conditions in the growth medium. Our results, however, are compatible with existing suggestions that there is a continuum ranging from loose, nonmycorrhizal root associations to fully mutualistic mycorrhizal associations, and that some fungal species may vary widely in status along this continuum depending on environmental conditions and host species present (Johnson *et al*. 1997, Perotto *et al*. 2002).

In our syntheses trials, we found significantly increased nitrogen quantities in plants in the fungal treatments (Figs 3, 4). We expect that this was due to the formation of an active mutualistic symbiosis, but we cannot exclude other explanations. For example, a diffusible growth-promoting compound could have been produced by the fungal species. Rudawska & Kieliszewska-Rokicka (1997) showed that ectomycorrhizal fungal strains with high auxin synthesizing capacity induced higher numbers of mycorrhizas than strains with lower capacity for auxin synthesis. This increased mycorrhiza formation, however, was not always accompanied by increased seedling growth. In general, little attention has been paid to the production of plant growth factors by ERM and endophytic fungi (Smith & Read 1997). Perhaps more important in interpretation of our experiments is that we cannot exclude that the production by endophytes of certain enzymes, e.g. polyphenol oxidases, played a role in mineralizing the organic nitrogen in our test systems, consistent with effects noted by Cairney & Burke (1998). In other *in vitro* experiments we performed, involving the degradation of soluble tannins in the so-called Bavendamm test, we found that some isolates, including *C. rhizophila* (CBS 109839) and ericaceous isolate PPO-E6, gave a positive response. Clearly, more research is needed to elucidate the mechanisms underlying the positive growth effect exerted by these endophytic fungi on host plants.

Though potentially mutualistic *P. fortinii*-like species have a very wide host range (Jumpponen & Trappe 1998, Ahlich *et al*. 1998, Addy *et al*. 2000, Grünig *et al*. 2002a), we did not find any such fungi in grass roots. No molecular evidence was found for a close relationship of any of the grass root fungi with species presently or historically known as *Phialophora* or *Phialocephala* (Fig. 1). Dark, septate hyphae were common in *D. flexuosa* roots and, correspondingly, 33 % of our cultures possessed dark hyphae (Table 5). These cultures failed to produce reproductive structures even after nine months, and their appearance differed from that of the *P. fortinii*-like cultures from ericaceous roots. The affinities of these fungal types, e.g. morphotype 1, are still unclear.

In our ericaceous fungal collection, *P. fortinii* and related *P. fortinii*-like species made up a substantial 22 % of isolates (Table 7). Jumpponen and Trappe (1998) reported that *P. fortinii* had been isolated repeatedly from ericaceous plants such as *V. myrtillus*, *V. vitis-idaea*, *C. vulgaris* and *E. nigrum*. *Phialocephala fortinii*-like species have not previously been reported from *E. tetralix*. Also, the positive growth effect of these fungi on both *D. flexuosa* and *C. vulgaris* seedlings has not previously been reported (compare Jumpponen 2001, Stoyke & Currah 1993). In the present study, we obtained numerous DSE cultures from ericoid sources but identified only the few that sporulated. It would be interesting in future to include additional isolates in molecular analyses to determine their relatedness to various known endophyte groups, including the *H. ericae* aggregate as delineated by Vrålstad (2002a).

The effects of simultaneous colonisation by two different mycorrhizal types are not clear. In *D. flexuosa*

roots, we saw that AMF structures were more abundant than dark hyphae (Figure 2). Dual colonisation has been found in a wide range of host plants, most of which conventionally were considered to host only AMF (Read 1991). A negative effect of dual colonisation can be suppression of the original mycorrhizal type. A field survey by Genney *et al*. (2001) revealed that *Nardus stricta* transplants within *C. vulgaris* swards developed little or no AMF colonisation. In our grass roots, however, we found no differences in AMF colonisation percentages among locations. The relatively dense colonization of grass roots by dark fungi at heathland and forest sites, contrasting with lower levels seen in grass monocultures, can be tentatively attributed to high inoculum densities produced by neighbouring non-grass host plants in heaths and forests. In our synthesis trials, where there was no competition from AMF, we found that grass roots were strongly colonised by the grass endophyte isolate PPO-G2, our chosen representative of the relatively pale coloured morphotype 2.

Whether AMF occur in ericoid plants is currently under debate. Davies *et al*. (2003) found that hair roots of epacrids were colonised by hyphae and vesicles typical of AMF. Earlier, Read (1991) pointed out that in the presence of a true AMF host the inoculum potential may be high enough to cause contaminating ingress into nearby ericaceous roots. In the present study, we found no AMF structures in our ericoid samples, even though grass AMF hosts were present at all collection sites.

In our ericaceous plants, the abundance of *O. maius* was low (Table 6). Johansson (2001) and Sharples *et al*. (2000), who also isolated ericoid endophytes from root pieces, obtained very low *O. maius* numbers, whereas they obtained *R. ericae* abundantly. A high abundance of *O. maius* from ericaceous roots, however, was shown in sites on Alberta and Vancouver Island, Canada, and northern Italy (Perotto *et al*. 1996, Hambleton & Currah 1997, Monreal *et al*. 1999). It appears that the abundance of *O. maius* isolates is related to particular site conditions (Perotto *et al*. 2002).

Our demonstration of a positive effect of ericoid endophytes on nutrient uptake in *D. flexuosa* can be considered preliminary. We realize that the role of Helotialean endophytes in the uptake of organic nitrogen by *D. flexuosa* should be clarified with isotopic labelling experiments. Also, as mentioned above, a true mycorrhizal association implies bidirectional transfer of nutrients and carbon (Smith & Read 1997, Read 2002). Nevertheless, the isolation of beneficial Helotialean endophytes from *D. flexuosa* encourages us to re-examine our ideas on organic nitrogen uptake by grasses in nutrient poor ecosystems, and to further investigate the nitrogen-related effects this fungal association might have on competition between *D. flexuosa* and ericaceous plants.

ACKNOWLEDGEMENTS

We thank Walter Gams for fungal identification. Frans Möller, Jan van Walsem and Henk van Roekel are acknowledged for their assistance in the field and the lab. We are grateful to Thom Kuyper and two anonymous reviewers for their valuable comments on the manuscript.

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