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

Jantineke D. Zijlstra[1]*, Pieter Van 't Hof[1], Jacqueline Baar[2], Gerard J.M. Verkley[3], Richard C. Summerbell[3], Istvan Paradi[2], Wim G. Braakhekke[1] and Frank Berendse[1]

[1]*Nature Conservation and Plant Ecology Group, Wageningen University, Bornsesteeg 69, 6708 PD, Wageningen, The Netherlands;* [2]*Mushroom Section of Applied Plant Research, Wageningen University and Research Centre, P.O. Box 6042, 5960 AA, Horst, The Netherlands;* [3]*Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands*

**Correspondence*: Jantineke D. Zijlstra jz@blgg.nl

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,