Small genetic differences between ericoid mycorrhizal fungi affect nitrogen uptake by *Vaccinium*

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Summary

- Ericoid mycorrhizal fungi have been shown to differ in their pattern of nitrogen (N) use in pure culture. Here, we investigate whether this functional variation is maintained in symbiosis using three ascomycetes from a clade not previously shown to include ericoid mycorrhizal taxa.
- *Vaccinium macrocarpon* and *Vaccinium vitis-idaea* were inoculated with three fungal strains known to form coils in *Vaccinium* roots, which differed in their patterns of N use in liquid culture. ^{15}N was used to trace the uptake of NH_4^+-N, NO_3^--N and glutamine-N into shoots.
- ^{15}N transfer differed among the three fungal strains, including two that had identical internal transcribed spacer (ITS) sequences, and was quantitatively related to fungal growth in liquid culture at low carbon availability.
- These results demonstrate that functional differences among closely related ericoid mycorrhizal fungi are maintained in symbiosis with their hosts, and suggest that N transfer to plant shoots in ericoid mycorrhizas is under fungal control.

Introduction

Ericaceous plants are widespread in Northern Hemisphere habitats such as heathland, tundra and the understory of boreal forests. A common feature of these habitats is their harsh edaphic conditions, characterized by acidic soils rich in recalcitrant organic matter and with a low availability of mineral nitrogen (N) and phosphorus (P) (Read, 1991; Cairney & Meharg, 2003). The success of ericaceous plants in these ecosystems is attributed to their symbiotic association with ericoid mycorrhizal (ERM) fungi (Cairney & Meharg, 2003; Read & Perez-Moreno, 2003).

Knowledge of the physiology of ERM symbioses is based mainly on studies with a strain of *Rhizoscyphus ericae* (formerly *Hymenoscyphus ericae*) pioneered by David Read and colleagues in the 1970s (Pearson & Read, 1973; Read & Stribley, 1973). However, with the advent of molecular techniques, the true diversity of fungi associated with ericaceous roots is beginning to become apparent (e.g. Bougoure *et al.*, 2007), and a single root system is now known to harbour several ERM fungal genotypes (Midgley *et al.*, 2002; Allen *et al.*, 2003). Does this genetic diversity confer functional diversity which could be advantageous to the symbionts in nutrient-poor habitats?

Cairney *et al.* (2000) showed significant variations in the pattern of N utilization in pure culture by various fungal strains isolated from hair roots of *Calluna vulgaris*. Five of these strains showed differing abilities to utilize organic and mineral N sources, and these abilities were affected by carbon (C) availability in a strain-specific manner (Grelet *et al.*, 2005). Whittaker & Cairney (2001) found differences in amino acid utilization by epacrid root endophytes. However, the relevance of these differences in fungal N and C nutritional patterns for the growth and N uptake of their host plant is unknown.

In this study, we first tested the hypothesis that fungal mycorrhizal strains, with different N use patterns in pure culture, confer different growth potentials and/or N uptake abilities on their host plants. To do so, we used a microcosm system to compare the growth and uptake of different N forms of seedlings of *Vaccinium macrocarpon* and *Vaccinium*

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vitis-ideae colonized by three closely related ERM strains known to differ in N metabolism (Grelet *et al.*, 2005). We also tested the hypothesis that the rate of uptake of different N forms by seedlings observed in microcosms was explained by the ability of their symbionts to utilize the same source of N in liquid culture, the underlying hypothesis being that N transfer to the plant shoot is controlled by the ERM fungal partner. To do this we compared the results from our microcosm experiment with those from an independent fungal culture experiment using the same fungal strains (Grelet *et al.*, 2005).

Materials and Methods

Identity of the fungal strains

Three fungal strains, AC2, AC21 and AC5, were isolated from *Calluna vulgaris* L. (Hull) hair roots. All three strains were shown to form typical ERM structures in epidermal root cells of *Vaccinium macrocarpon* Ait. by Sharples *et al.* (2000). The three strains differed in their relative ability to utilize various N sources at high or low C availability (Grelet *et al.*, 2005). The strains were subsequently deposited in Centre for Agriculture and Bioscience International (CABI), with accession codes IMI 392567 (AC2), IMI 392568 (AC21) and IMI 392570 (AC5). Pure culture mycelium was processed for DNA extraction and internal transcribed spacer (ITS) sequence analyses as described in Villarreal-Ruiz *et al.* (2004), using primers ITS1f (Gardes & Bruns, 1993) and ITS4 (White *et al.*, 1990). Searches for similar sequences deposited in GenBank were conducted using Fasta3 at the European Bioinformatics Institute website (http://www.ebi.ac.uk/).

The alignment (available upon request) included complete sequences of the ITS1, 5.8S, and partial ITS2 (truncated before the last 18 bp) regions of the rDNA gene cluster. Reference sequences were downloaded from GenBank for (1) the four main clades of the *Rhizoscyphus ericae* aggregate (as defined by Hambleton & Sigler, 2005), (2) a range of species currently assigned to the Helotiaceae, Loramycetaceae, Dermatiaceae, Hyaloscyphaceae, Leotiaceae and Hemiphacidiaceae (Wang *et al.*, 2006; Hibbett *et al.*, 2007), and (3) the single similar sequence retrieved using a Fasta search which corresponded to a vouchered fungal specimen. Sequences of *Rhabdocline pseudotsugae* and *Meria laricis,* placed either in the Rhytismatales or in the Heliotales (Eriksson & Hawksworth, 1993; Gernandt *et al.*, 2001; Wang *et al.*, 2006), were also included. *Meloderma desmaziereni* and *Elytroderma deformans* (Rhytismatales) were used as outgroups. Phylogenetic analyses were conducted with PAUP* 4.0b10 (Swofford, 2003) using the heuristic search option with 1000 replicates and the random stepwise addition option, 'TBR' branch-swapping, MULPARS, and the collapse zero length branches options switched on. For the bootstrap analysis, the heuristic search option was used with 1000 bootstrap replicates and 'Maxtrees' set to 1000. Percentage sequence identity (SI) was calculated using the sequence identity matrix module in BioEdit (version 7.0.9; Hall, 1999).

Microcosm experimental design

Two *Vaccinium* species, differing in their geographical distribution and natural habitat, were used: *Vaccinium vitis-idaea* L., the European cowberry, and *V. macrocarpon* Ait., the American cranberry. Seedlings were inoculated with strain AC2, AC21 or AC5 (mycorrhizal (M)) or with heat-killed inoculum as a nonmycorrhizal control (NM). Plant species, ERM fungal strains and N treatments were combined in a factorial design within four replicate blocks. Nitrogen amounts and concentrations supplied to the plants were equal in all N treatments. Three forms of N were tested: glutamine (simple organic N), ammonium (reduced mineral N) and nitrate (oxidized mineral N). In the main N treatments, N was supplied at natural abundance (unlabelled) for 21 wk (period 1), and then enriched with ^{15}N (labelled N) for the last 48–69 h of the experiment (period 2) to assess the rate of N uptake. These treatments were paired with control treatments where the same source of unlabelled N was supplied in period 1, but N supply in the last 48–69 h was withheld to determine background ^{15}N enrichment of plant shoots. We also included a treatment where we did not supply any N to the microcosms (treatment 'no N'), to assess plant growth response to mycorrhizal strains where peat was the only source of N available. In addition, two subsidiary treatments were included where unlabelled ammonium was switched to either labelled glutamine or labelled nitrate. These last two treatments were designed to assess whether uptake of labelled N depended on the plant size and N status at the end of period 1. All N treatments were applied within 30 min to all microcosms within each replicate block.

Microcosm preparation

Surface peat was collected from beneath an unfertilized, mixed *Vaccinium myrtillus* L./*Calluna vulgaris* stand in Aberdeenshire (north-east Scotland, Ordnance Survey grid reference NJ033735), air-dried, sieved (< 2 mm), gamma-irradiated and rinsed in sterile water under aseptic conditions, before being combined with vermiculite (diameter < 2 mm) in a 1 : 3 ratio. This substrate (40 cm^3) together with 5 cm^3 of N- and glucose-free nutrient solution (see next paragraph) was added to cylindrical glass vials (2.7 cm diameter × 17 cm height), before sterilization at 121°C and 210 kPa for 1 h. The vials were fitted with a sterile cotton-wool plug and a loose aluminium cap, which allowed air diffusion.

N sources and glucose were filter-sterilized and added to each tube after autoclaving. The nutrient solution provided 22 mol N m^{-3} at natural abundance as ammonium, nitrate or glutamine, or no N, 6.3 mol P m^{-3}, 8.9 mol K m^{-3},

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0.55 mol glucose m^{-3} and a complete set of micronutrients (Supporting Information Table S1). Spare tubes were prepared to check that the pH in the 'soil solution' was within the range 4.5–5. In total, we added 1.85 mg of unlabelled N per microcosm in 6 cm^3 of nutrient solution.

After isolation (Sharples *et al.*, 2000), isolates were stored on modified Melin Norkrans (MMN) agar at 4°C (Marx & Bryan, 1975). Three months before the start of the experiments, the isolates were subcultured every 4 wk and incubated at 20°C in the dark. Two weeks before planting, each microcosm was inoculated with an inoculum disc (3 mm diameter) cut from the leading edge of three actively growing colonies of the same isolate. The nonmycorrhizal treatment was set up by inoculating each microcosm before autoclaving, with an inoculum disc of each of the three isolates.

Seeds of *V. macrocarpon* and *V. vitis-idaea* were surface-sterilized (H_2O_2 at 10% v/v for 20 min) and germinated in Petri dishes containing modified Ingestad solution (Ingestad, 1973; Grelet *et al.*, 2001) solidified with 0.8% agar, to which 1 mg m^{-3} gibberellic acid had been added. The seedlings were allowed to grow in the Petri dishes at ambient temperature and light for a minimum of 2 months. Three sterile seedlings were then transplanted in aseptic conditions to each microcosm, 2 wk after fungal inoculation. Microcosms were arranged in four replicate randomized blocks in a growth chamber (photoperiod 16 h, relative humidity 80%, temperature 25 : 15°C day:night measured inside the microcosms and photosynthetically active radiation (PAR) 200 µmol m^{-2} s^{-1}) for the remainder of the experiment (21 wk). A total of 288 microcosms were set up. Each inoculum (4) × plant species (2) × N treatment (9) combination had four replicate microcosms, each microcosm containing three seedlings.

^{15}N-labelling, harvest and post-harvest measurements

Before harvest (48–69 h), 1 cm^3 of nutrient solution was added to each microcosm, to provide 30.8 µg of labelled N (enriched with ^{15}N at 98 atom %) per microcosm in the form of ammonium, glutamine or nitrate, as appropriate (Table S2). Microcosms assigned to the paired control treatments received 1 cm^3 of N-free nutrient solution. These treatments were designed to measure background $^{15/14}$N in plant shoots. At harvest, the seedling shoots were cut off and immediately frozen in liquid nitrogen. Shoot-free microcosms were kept for a further 48 h in a cold room (5°C) until the root system was harvested and root fresh weight measured. One microcosm per treatment combination (plant species × N treatment × mycorrhizal inoculum) was randomly selected and its root system checked to confirm the presence of fungal coils in epidermal cells. The level of infection was not measured.

The dry weight of the root systems harvested to assess mycorrhizal infection was estimated, using a conversion factor between fresh and dry weight calculated for the three other replicates within each treatment. All other root systems were dried at 60°C and weighed. Root N content and $^{14/15}$N ratio were not measured, on the grounds that it was impossible to distinguish between plant and fungal N. The freeze-dried shoots were weighed and their N content and $^{14/15}$N ratio analysed using a NA1500 Elemental Analyser (Carlo Erba, Milan, Italy) linked to a DELTA plus Advantage mass spectrometer (Thermo Finnigan, Bremen, Germany).

Fungal culture experiment

The data for fungal biomass in pure culture were taken from Grelet *et al.* (2005), where details of growth conditions, harvest and analytical procedures can be found. Briefly, a factorial design was used to grow five mycorrhizal strains, including AC2, AC21 and AC5, in liquid media containing ammonium-, glutamine- or nitrate-N at starting concentration of 7.5 mol N m^{-3} with a C:N ratio of either 39 : 1 (high C availability (HC)) or 9 : 1 (low C availability (LC)). Fungal biomass was collected after 30 d of growth in the dark at 20°C and dried at 60°C for 48 h before weighing of dry mass.

Data analyses

Seedling survival was expressed as the mean number of seedlings per microcosm alive after 2 wk (ranging from 0 to 3). Seedling persistence was expressed as the proportion of these surviving seedlings that were still alive at the end of the 21-wk period. Growth and N use were assessed on a per seedling basis by calculating the mean shoot and root biomass, shoot N concentration and content of the persisting seedlings in each replicate microcosm. These data were used for statistical analyses of treatment effects on growth and N use per seedling. Preliminary analyses showed that growth and N status at the end of the experiment were not affected by the supply of labelled N, because growth and N uptake during the last 48–69 h of the experiment were very low compared with values obtained during the previous 21 wk. Therefore, the effect of unlabelled N source on plant growth (shoot dry weight) and N status (shoot N concentration and content) was assessed across all treatments irrespective of the source of labelled N supplied, using REML (REsidual Maximum Likelihood) in GENSTAT release 7.2 (VSN International, Hemel Hempstead, UK), to allow for unequal number of replicates per treatment combination when treatments were combined.

After harvest, ^{15}N abundance in plant shoots was used to calculate the amount of labelled N taken up and transferred to plant shoots in each N treatment. Because the source of unlabelled N affected seedling growth and N status, treatment differences were present before the start of the labelling period. For each microcosm, we calculated a variable corresponding to the amount of unlabelled N not recovered in the shoot tissues (i.e. remaining in the soil substrate, in the root system or in extramatrical fungal hyphae) by subtracting shoot N content from the total amount of unlabelled N added. This

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variable was used as a covariate for the analysis of variance of the rate of labelled N appearance in the plant shoot, to factor out treatment differences arising from differences in N uptake before the addition of labelled N.

To assess mycorrhizal treatment effects, a two-step approach was taken. First, the effect of mycorrhizal colonization *per se*, that is, comparing M and NM plants, was tested. Secondly, if significant differences between M and NM were found, the effect of mycorrhizal strain was tested with the data set restricted to mycorrhizal plants only.

Relationships between fungal biomass production in pure culture and shoot N uptake in the re-synthesis experiment were assessed using a weighted linear regression model (Ripley & Thomson, 1987). This regression approach, based here on mean values for each treatment combination, was considered appropriate as it takes into account the standard errors associated with mean values of x as well as those associated with the y variables. The relevant data from both the pure culture and microcosm experiments had previously been averaged with respect to treatment combinations, as a means of addressing the issue of regression variables being derived from different experimental material. Regression modelling was carried out in S-Plus (Venables & Ripley, 2002). Relationships were taken to be significant only when points were not organized in clusters and slope coefficients were significantly different from 0 at $P < 0.01$ (t-test). Because of the small size of the data set used ($n = 9$), we chose a lower significance threshold ($P < 0.01$) to reduce the probability of scoring for significant effect when there was none.

Data were transformed to their decimal logarithm or square-root as required, to comply with assumption of heterogeneity of variance and normal distribution of the residuals in all analyses. Graphic illustrations are based on back-transformed means, where appropriate. Log_{10} means were back-transformed after addition of 1.15 × mean square of residuals.

Results

Fungal strains and formation of ERM structures

Strains AC2 and AC21 had identical ITS sequences (over the complete ITS1*5.8S*ITS2 region). AC5 shared 92% SI with AC2 and AC21. Sequences were deposited in GenBank under accession numbers FM180476 (AC2), FM180477 (AC21) and FM180480 (AC5).

The strains were previously placed in the *R. ericae* aggregate (Sharples *et al.*, 2000), the main group of Helotiales known to form ERM associations. Our phylogenetic analysis now places the three strains in the Helotiales, but outside the *R. ericae* aggregate, with 97% and 100% bootstrap support for the AC21/AC2/AC5 cluster and the *R. ericae* aggregate, respectively (Fig. 1). The two groups share a maximum of 77.2% SI with each other. The AC21/AC2/AC5 cluster also contains an unknown Salal endophyte (AF149077) and *Hyphodiscus hymeniophilus* (DQ227264), with which they shared 88.3–91.6% SI.

Typical ERM fungal coils were observed in the hair roots from microcosms given live inoculum of all three strains. No fungal structures were observed in roots from the control microcosms. These are henceforth referred to as mycorrhizal (M) and nonmycorrhizal (NM) treatments, respectively.

Seedling survival and persistence

Seedling survival, that is, the mean number of living seedlings per microcosm 2 wk after planting (minimum 0, maximum 3), was not affected by plant species or N source, but depended on the mycorrhizal treatment (see Table S3 for P-values). Fewer seedlings survived with AC21 (2.3 ± 0.1 surviving seedlings per microcosm) than AC2 (2.6 ± 0.1) ($P < 0.05$). Seedling survival with AC5 (2.5 ± 0.1) was not significantly different from that with either AC2 or AC21. The survival of nonmycorrhizal seedlings (2.0 ± 0.10) was significantly lower than that of mycorrhizal seedlings ($P < 0.001$).

Between 25 and 89% of the seedlings persisted to the end of the 21-wk period, depending on plant species ($P = 0.006$) and N source ($P = 0.001$) (Fig. 2a). In total, we had 72 treatment combinations. At the end of the experiment, six treatment combinations were down to three replicate microcosms (out of the four initially set up) because all three seedlings died in one of the microcosms. However, in all remaining treatment combinations, all four replicate microcosms contained between one and three healthy seedlings. Mycorrhizal infection improved seedling persistence ($P = 0.01$), irrespective of fungal strain, but the two plant species reacted differently (Fig. 2a). Persistence of *V. macrocarpon* increased in M compared with NM seedlings, irrespective of the source of N. For *V. vitis-idaea*, mycorrhizal infection did not affect seedling persistence, except when N was supplied as glutamine, in which case more M seedlings persisted compared with NM seedlings. In the treatment with no N, persistence of *V. vitis-idaea* was 2.7 times greater than that of *V. macrocarpon* (Fig. 2a).

Plant biomass and N concentration and content

The shoot biomass of *V. macrocarpon* M seedlings was the same as, or greater than, that of NM seedlings, while the reverse was true for *V. vitis-idaea* (Fig. 2b). The response of root growth to mycorrhizal infection showed a similar pattern, with enhanced root biomass in mycorrhizal *V. macrocarpon*, but similar or reduced root biomass in mycorrhizal *V. vitis-idaea* (data not shown). Shoot N concentration was reduced in M seedlings of both plant species, but to a greater extent in *V. macrocarpon* ($P < 0.05$; Fig. 2c). Consequently, N content in mycorrhizal seedlings of *V. vitis-idaea* was only 10–33% of that in their NM counterparts, while no significant difference was found between M and NM seedlings of *V. macrocarpon*,

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AF069505 ***Rhizoscyphus ericae*** (Read 101)
AJ292200 ***Melinomyces vraolstadiae*** (ARON2917.S)
AF011327 ***Cadophora finlandica*** (FAG15)
AY838783 ***Meliniomyces variabilis*** (UAMH5900)
AY524845 ***Phialocephala sphaeroides*** (UAMH10294)
AF214579 ***Phialocephala fortinii*** (UAMH9525)
AJ430396 ***Hymenoscyphus fructigenus*** (ARON3264.H)
AJ430397 ***Cyathicula coronata*** (ARON3093.H)
AF141157 ***Dermea hamamelidis*** (CBS 137.46)
AF141167 ***Pezicula heterochroma*** (CBS 199.46)
AF141199 ***Pezicula ocellata*** strain (CBS 949.97)
AF141164 ***Dermea acerina*** (CBS 161.38)
DQ227264 ***Hyphodiscus hymeniophilus*** strain (CBS 602.77)
strain AC2 (IMI292567)
strain AC21 (IMI392568)
AF149077 Salal mycorrhizal fungus (UBCtra179)
strain AC5 (IMI392570)
AF141161 ***Neofabraea malicorticis*** (CBS 141.22)
U57492 ***Cistella acuum*** (JHH3966 NYS)
AJ430218 ***Lachnum pygmaeum*** (ARON3248.P)
U58638 ***Lachnum controversum***
AY144541 ***Leotia lubrica*** (strain 2Eng)
AY144535 ***Leotia viscosa*** (strain 3Chi)
AY144534 ***Microglossum viride***
AY144533 ***Microglossum rufum***
U92298 ***Meria laricis*** (isolate 1)
U92290 ***Rhabdocline pseudotsugae subsp pseudotsugae*** (isolate 2)
AF203470 ***Meloderma desmazieresii*** (CBS 612.84)
AF203469 ***Elytroderma deformans*** (CBS 181.68)
50 changes
Helotiales
Outgroups: Rhytismatales

Fig. 1 One of the three most parsimonious trees obtained with the heuristic search algorithm in PAUP*, showing the placement of strains AC2, AC21 and AC5 outside the *Rhizoscyphus ericae* aggregate. *Meloderma desmaziereni* and *Elytroderma deformans* (Rhytismatales) were used as outgroup taxa. Numbers above horizontal bars indicate the per cent bootstrap support derived from bootstrap analysis using the full heuristic search option and 1000 replicates.

irrespective of the source of N supply (Fig. 2d). There was no significant effect of fungal strain on the shoot and root biomass, or shoot N concentration of mycorrhizal plants ($P > 0.05$). This was also the case in the microcosms given no added N, where the only source of N was that derived from the peat.

Recovery of labelled N in plant shoots

^{15}N appearance in shoots was expressed as the amount of labelled N recovered per unit shoot mass per hour (Fig. 3). When the N source was kept constant throughout the experiment, that is, when the source of both unlabelled and labelled N was the same, the two plant species exhibited similar rates of N uptake ($P > 0.05$), and there were no significant interactions between plant species and either mycorrhizal infection or N source. Hence the data were pooled across the two plant species for all remaining statistical analyses. N uptake differed significantly between N sources ($P < 0.001$), mainly because, when N was supplied as nitrate, uptake was at least 2.5 times slower than when N was supplied as glutamine or ammonium (Fig. 3). Significant differences were found between M and NM seedlings, and among fungal strains within M seedlings ($P < 0.001$).

When N was supplied as glutamine or ammonium, uptake was 50% higher into seedlings inoculated with AC2 and AC21 than into NM seedlings or those inoculated with AC5 (Fig. 3). When N was supplied as nitrate, uptake was similar in all mycorrhizal treatments.

In these analyses, we included a covariate approximating the proportion of unlabelled N supplied that had been taken up by the plants before labelling. This covariate was significant ($P < 0.01$). Therefore, we needed to check directly that treatment effects occurring during the labelling period (period 2) did not simply emerge from treatment differences induced

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Table 1 Effect of nitrogen (N) sources in periods 1 and 2 on recovery of labelled N in *Vaccinium* plant shoots

Unlabelled N source in period 1 (N1 = 21 wk)	Labelled N source in period 2 (N2 < 3 d)	Recovery of ^{15}N in shoots[1]
Glutamine	Glutamine	0.64 ± 0.04[a2]
Ammonium	Glutamine	0.63 ± 0.04[a]
Nitrate	Nitrate	0.23 ± 0.04[b]
Ammonium	Nitrate	0.35 ± 0.04[b]

[1]Data are means (± SE) of four replicates for the rate of labelled appearance in the plant shoot (transformed to $\log_{10}$), obtained from two-way ANOVA (n = 64). Previous four-way ANOVA showed no significant differences between plant species, and no significant effect of the interaction between N1 and strains, and hence the data are averaged across strains and plant species.
[2]Means with different letters within the column are different at P < 0.001.

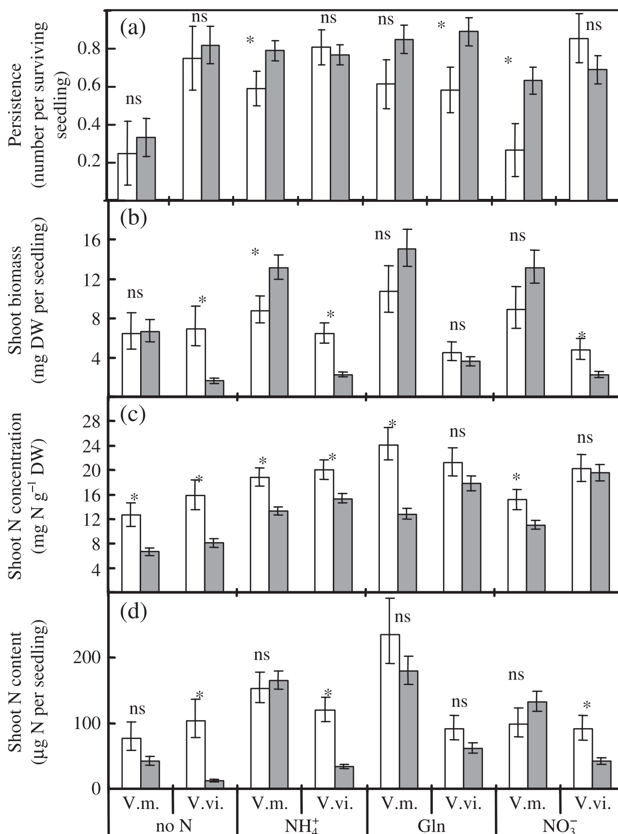

Fig. 2 Seedling persistence (a), shoot biomass (b), nitrogen (N) concentration (c) and N content (d) of nonmycorrhizal (open bars) or mycorrhizal (closed bars) seedlings of *Vaccinium macrocarpon* (V.m.) and *Vaccinium vitis-idaea* (V.vi.) supplied with ammonium (NH_4^+), glutamine (Gln), nitrate (NO_3^-) or no added N (no N). Data are means (columns) ± SE (vertical bars). ns, not significant; *P < 0.05.

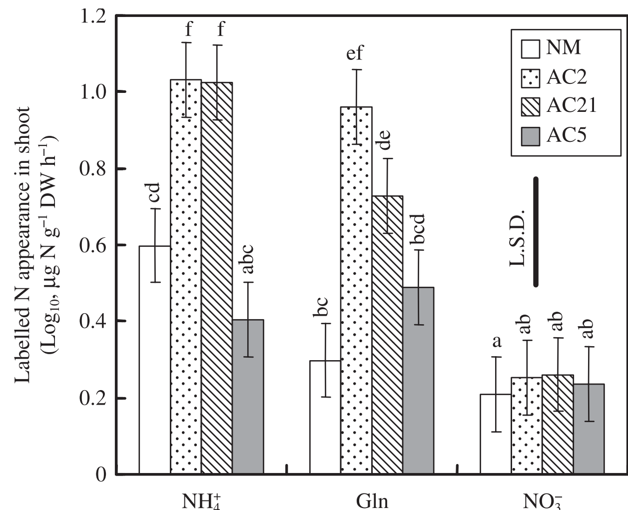

Fig. 3 Rate of appearance of labelled nitrogen (N) in *Vaccinium* plant shoots after 48 h of exposure to ^{15}N. Data are means (columns) of eight replicates ± SE (vertical bars), averaged across the two plant species, nonmycorrhizal (NM) or infected with strain AC2, AC21 or AC5, and supplied with N as ammonium (NH_4^+), glutamine (Gln) or nitrate (NO_3^-) throughout the experiment. Means with different letters are significantly different at P < 0.05. The least significant difference between means is given at P < 0.05.

before the labelling period (period 1). To do so, we compared uptake of labelled N in seedlings supplied with labelled N in period 2 as either glutamine or nitrate, after unlabelled N had been supplied in period 1 as ammonium or as the same source of N as that used for ^{15}N labelling (glutamine or nitrate, respectively). There was no effect of plant species or mycorrhizal status (data not shown) and the source of N in period 1 (N1) did not affect uptake in period 2 (Table 1). Furthermore, there was no significant interaction between N1 and mycorrhizal strain (AC21, AC2, AC5 and NM). This confirms that N source- and strain-induced differences in the rate of N transfer to the plant shoot measured in period 2 were not an indirect effect of treatment in period 1.

Relationship between fungal growth in pure culture and N uptake by mycorrhizal seedlings

For both plant species there was a highly significant relationship between labelled N appearance in plant shoots and the growth of the corresponding fungal isolate in liquid culture at low C:N ratio (Fig. 4). There was no relationship between labelled N in shoots and fungal growth at higher C:N ratio. Our

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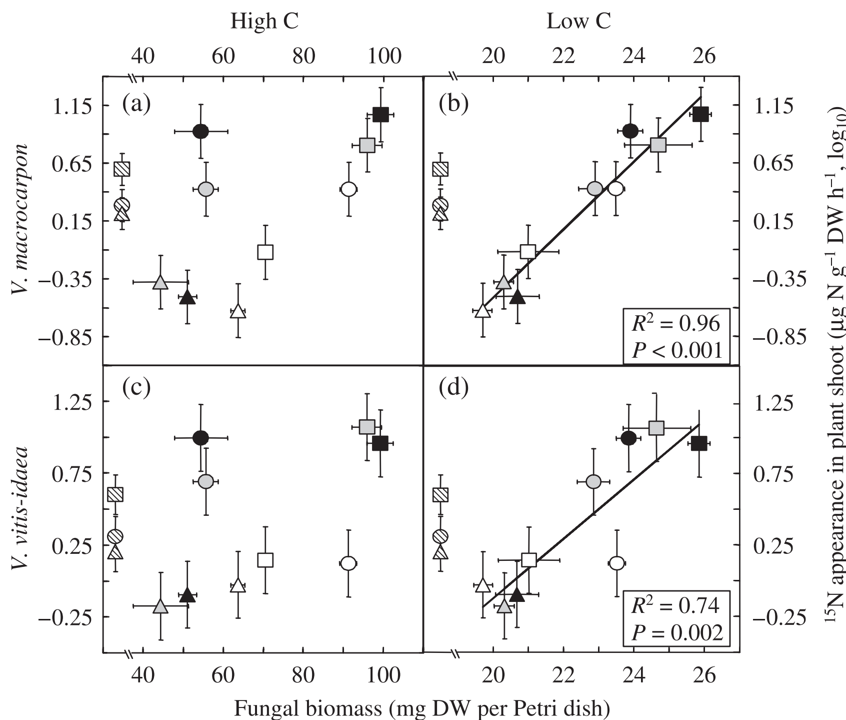

Fig. 4 Relationship between growth of mycorrhizal isolates in pure culture and rate of nitrogen (N) uptake by mycorrhizal *Vaccinium* seedlings. Fungal isolates were grown in pure culture at either high (a, c) or low (b, d) carbon availability. N uptake was measured for plants of *Vaccinium macrocarpon* (a, b) and *Vaccinium vitis-idaea* (c, d). Nitrogen was supplied as ammonium (squares), glutamine (circles) or nitrate (triangles). Values of N uptake measured in nonmycorrhizal seedlings (dashed symbols) are plotted for comparison with mycorrhizal isolates AC2 (black symbols), AC21 (grey symbols) and AC5 (open symbols). The *F* probability for the significance of the regression slope (*P*-value) and the percentage of variance explained by the data (R^2) are given when regressions are significant at $P < 0.01$, and were calculated using a weighted linear regression model accounting for standard errors associated with both *x* and *y* variables.

results show a significant quantitative relationship between fungal N use in conditions of C limitation and rate of N transfer to the plant shoot within the mycorrhizal plant–fungus association.

Discussion

Phylogenetic placement of strains AC2, AC21 and AC5

The isolates used in this study bear the strain identifiers AC2, AC21 and AC5 assigned by Sharples *et al.* (2000) and were used under those identifiers in our previous pure culture experiment (Grelet *et al.*, 2005) and in the microcosm experiment reported here. These fungi were originally placed within the *R. ericae* aggregate on the basis of restriction fragment length polymorphism (RFLP) profiles (Sharples *et al.*, 2000). However, based on the rDNA-ITS sequences reported here, it is clear that the isolates AC2, AC21 and AC5 do not belong to the *R. ericae* aggregate. Nevertheless, all three isolates clearly behave as ericaceous endophytes because they formed coils in the epidermal root cells of *Vaccinium* spp., and influenced plant N uptake. Two other strains used by Grelet *et al.* (2005), AC1 (IMI392566:FM180479) and AC14 (IMI392569:FM180478), shared 98.7% and 99.7% SI with AC2/AC21 and AC5, respectively. The results reported by Grelet *et al.* (2005), and here, therefore apply not to *R. ericae* but to this new group of ericoid endophytes.

On the basis of their ITS sequences AC2, AC21 and AC5 appear to be related to *Hyphodiscus hymeniophilus* (anamorph *Catenulifera rhodogena*). The position of *Catenulifera* within the Helotiales cannot fully be resolved based on sequences of the nuclear Large and Small Subunits rDNA (Untereiner *et al.*, 2006), but some isolates are close to Dermateaceae and Hyaloscyphaceae. Our ITS-based phylogenetic analysis also grouped AC2, AC21, AC5 and *H. hymeniophilus* with other Dermateaceae, but with poor bootstrap support (66%). AC2, AC21 and AC5 are likely to be in the Helotiales, but their exact phylogenetic position cannot be resolved at this stage.

The ascomycetes involved in ERM symbioses have traditionally been thought to be restricted to the *Rhizoscyphus* aggregate and *Oidodendron* spp. A few other ascomycetes (e.g. *Capronia* and *Geomyces*) also form coils in hair roots of Ericaceae (Dalpé, 1989; Allen *et al.*, 2003; Vohnik *et al.*, 2007) but, as far as we know, our study is the first to demonstrate that 'nontraditional' ascomyceteous endophytes can transfer N to their ericaceous host plants.

The two plant species responded differently to mycorrhizal infection

Mycorrhizal infection inhibited shoot growth of *V. vitis-idaea*, but stimulated that of *V. macrocarpon*, irrespective of the isolate or the N source. We observed typical ericoid coil-like structures in the roots of both plant species, and fungal infection increased the rate of ^{15}N transfer to the shoots of both species. Our data suggest that the three strains function as ERM partners with both *Vaccinium* species, bearing in mind the functioning of mycorrhizas along the 'mutualism–parasitism continuum'

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(Johnson *et al.*, 1997). Obviously, the particular experimental conditions might have triggered differences between plant species in their relationship with these fungal endophytes, at least at the seedling stage. However, the growth depletion observed in *V. vitis-idea* but not *V. macrocarpon* may also reflect differences in their inherent ability to up-regulate photosynthesis in response to mycorrhizal infection, demonstrated in several endo- and ectomycorrhizal host plants (e.g. Louche-Tessandier *et al.*, 1999; Loewe *et al.*, 2000) but to our knowledge never in ERM species. Our results emphasize the need for caution when assessing the potential 'benefit' of fungal partners on the sole basis of fungal effect on plant biomass or N content increase, especially when inherent species-specific plant growth patterns are ignored.

Mycorrhizal genotypes influence seedling survival and N transfer to plant shoot

Mycorrhizal infection increased seedling survival to different extents depending on the fungal strain used for inoculation. Strikingly, the main difference occurred between AC2 and AC21, the two strains with identical rDNA-ITS sequences. At the time of planting, the seedlings had very small root systems, and thus it is possible that the strain-induced differences were mediated via fungal effects on root growth and/or rates of root colonization, which we did not measure.

The rate of appearance of labelled N in *Vaccinium* plant shoots depended strongly on mycorrhizal strain. In ectomycorrhizas, the kinetics of N uptake as amino acids or mineral N also appears to depend largely on the fungal species/strain hosted in the root system (e.g. Wallenda & Read, 1999; Wallander *et al.*, 1999; Plassard *et al.*, 2002). Plassard *et al.* (2002) reported differences in net ion fluxes in *Pinus pinaster* roots associated with ectomycorrhizal isolates of different genera. Our study is the first demonstration in ERM symbiosis that plant N acquisition is influenced by the fungal symbiont harboured in its root system in a strain-specific manner and that differences can be observed between strains that are genetically closely related.

Our data confirm that the N uptake kinetics in ERM plants is strongly influenced by that of the fungal symbiont. In our experimental conditions, this was evident when N was supplied as ammonium or glutamine, but others have also reported fungal influence on plant N uptake for a range of amino acids (Sokolovski *et al.*, 2002) and nitrate (Kosola *et al.*, 2007). The apparent strain-specific effect of N transfer to the plant shoot held for both plant species, despite contrasting responses to mycorrhizal infection. These results are consistent with an uptake model similar to that emerging from studies of phosphate uptake by arbuscular mycorrhizal symbioses, where a symbiotic pathway can outweigh and sometimes cause the complete inactivation of the plant uptake pathway, independently of the plant growth response to mycorrhizal infection (Smith *et al.*, 2003, 2004; Javot *et al.*, 2007).

There were no effects of mycorrhizal strain on plant shoot N contents, despite the clear differences in N transfer. Scagel (2005) compared the growth responses of *V. corymbosum* cultivars to infection by one of three ERM fungal strains: *Pezizella ericae*, *Hymenoscyphus ericae* and *Oidiodendron griseum*. The author did not observe any difference in plant N content but plant uptake of boron and zinc was significantly affected by mycorrhizal species ($P < 0.05$; C. Scagel, pers. comm.). There is an obvious heterogeneity of time-scale between measurement of nutrient uptake via plant or shoot nutrient content and our short-term labelled-N tracer study. The rate of labelled N transfer to the plant shoot was assessed over 2–3 d. By contrast, shoot N content reflected N allocation to seedling shoots over the entire experimental period (21 wk), which depended upon the sink strength exerted by the shoots as they grew and was likely to be regulated by many other factors than N uptake *per se*. In addition, all three isolates influenced host growth and N uptake in a similar manner when autoclaved peat was the only source of N. Therefore, the long-term effect of strain-specific differences in uptake and transfer of ammonium, glutamine or nitrate may have been masked by a overall lack of strain specificity in the use of peat-derived N.

There was no obvious indication that plant growth was N limited, as shoot N concentrations fell within the range reported from other field and controlled environment experiments (Davenport & Provost, 1994; Grelet, 2001; Olsrud *et al.*, 2004). However, fungal and/or plant growth and N uptake were probably limited by C availability, as indicated by the negative effect of mycorrhizal infection on shoot growth and N content of *V. vitis-idaea.* Photosynthesis may have been hindered by slow CO_2 diffusion into the vials, a common flaw of sterile microcosm systems. Light intensity is unlikely to have caused an unusual limitation to photosynthesis, as *Vaccinium* photosynthesis saturates in the region of 300 μmol m^{-2} s^{-1} PAR (Kolari *et al.*, 2006), only 100 μmol m^{-2} s^{-1} above the intensity applied in our experimental growth cabinet.

The quantitative relationship between fungal N use and N transfer to the plant shoot depends on C availability to the fungal partner

We found a significant quantitative relationship between fungal N use in pure culture and labelled N transfer to the plant shoot, for both plant species. A remarkable feature of this apparent direct correlation is that it held only when fungal N use was assessed in a growth medium low in C availability. Was this a consequence of a potential C limitation to plant and fungal growth in our microcosm systems? Possibly, but this would indicate that pure culture studies, despite widespread criticisms, can yield relevant information on the C/N physiology of ERM symbioses, providing that levels of C and nutrient availability are comparable between pure culture and microcosms/field studies. The relevance of pure culture studies was argued by Fransson *et al.* (2007), who

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Table 2 Relationship between fungal radial growth in pure culture of six ectomycorrhizal isolates (four *Paxillus involutus* and two *Suillus bovinus* isolates) and total ^{15}N uptake by *Pinus sylvestris* colonized by the corresponding isolate (data from Wallander *et al*., 1999)

Glucose concentration[1]	Ammonium concentration[2]	R^2 (unweighted regression)	Overdispersion parameter (weighted regression)[3]	R^2 (weighted regression)[3]	Significance[4]
High	High	0.46	10.1	0.88	ns
High	Low	0.45	22.5	0.78	ns
Low	High	0.79	0.88	0.99	$P < 0.001$
Low	Low	0.52	12.2	0.64	ns

[1]Glucose concentrations in pure culture were set at 6 (high) and 3 (low) g l^{-1}. [2]Ammonium concentrations were set at 150 (high) and 50 (low) mg l^{-1}. Fungal radial growth was measured after 30 d of culture on modified Melin Norkrans (MMN) agar overlaid with cellophane. ^{15}N uptake by ectomycorrhizal pine seedlings growing in peat/sand mixture was measured over 3 months. ^{15}N was supplied as NH_4^+ within a nutrient solution contained in a vial only accessible to fungal hyphae. For a full detailed description of the experimental procedures, see Wallander *et al*. (1999). [3]Weighted regression analyses as in Fig. 4. [4]Significance levels given at $P < 0.01$.

found that the responses of 17 ectomycorrhizal fungal isolates to increased C availability in liquid culture mapped onto what was known about the ecology of the species concerned. Wallander *et al.* (1999) ran a series of experiments similar to ours, to investigate functional variations among multiple isolates of ectomycorrhizal *Paxillus involutus* and *Suillus bovinus.* They reported strain-to-strain variations in fungal growth performance under contrasting levels of C (glucose) and N (ammonium) availability and showed that these differences were reflected in the uptake of ^{15}N-labelled ammonium by *Pinus sylvestris* colonized by each of these isolates growing under light intensities similar to those experienced by pine seedlings in semi-closed canopies. We recalculated their data to test whether a quantitative relationship between fungal N use in pure culture and ^{15}N uptake by the symbiosis also applied in their experiment. The relationship was indeed highly significant, but only when fungal growth was measured under a 'low' level of C availability and a 'high' level of N availability (Table 2), corresponding to a C:N ratio of 8 : 1, similar to the ratio used in our experiment (9 : 1) in our low C treatment.

Linking fungal genetic diversity to function at the symbiosis level

The fungal isolates used in this study were closely related, with two isolates sharing 100% SI in the ITS region of the rDNA gene cluster. The rDNA-ITS is currently the region of choice for 'defining' fungal species, and assessing diversity of fungal communities in ecological studies. Our results clearly demonstrate that functional differences exist within identical ITS types and emphasize the need for comparison to be made at the genotype level.

Our results also imply that, when the root system is colonized by a single fungal isolate, N uptake kinetics can be dominated by that of the fungal partner. However, ericaceous root systems simultaneously harbour many different fungal strains (Allen *et al.*, 2003; Bougoure *et al.*, 2007), with possibly one or a few dominant isolates (Midgley *et al.*, 2002; Bougoure & Cairney, 2005a,b). Should these isolates differ in their N use, functional differences amongst fungal partners may provide a competitive advantage to the symbiosis in an environment where qualitative and quantitative fluctuations of N supply occur at both temporal and spatial scales.

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Supporting Information

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Table S1 Chemicals and concentration for nutrient solutions used in the different nitrogen (N) treatments

Table S2 Experimental treatments

Table S3 χ^2 probabilities for the effect of mycorrhizal infection/strain and interaction with plant species and nitrogen (N) source on plant growth parameters

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