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Fungal colonization patterns and enzymatic activities of peatland ericaceous plants following long-term nutrient addition

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ABSTRACT

Northern peatlands are often dominated by ericaceous shrub species which rely on ericoid mycorrhizal fungi (ERM) for access to organic sources of nutrients, such as nitrogen (N) and phosphorus (P), and host abundant dark septate endophytes (DSE). Relationships between hosts and fungal symbionts may change during deposition of anthropogenic N and P. We studied the long-term effects of N and P addition on two ericaceous shrubs, *Calluna vulgaris* and *Erica tetralix*, at Whim Bog, Scotland by analyzing fungal colonization of roots, enzymatic activity, and fungal species composition. Unexpectedly, the frequency of typical ERM intracellular colonization did not change while the occurrence of ERM hyphae tended to increase and DSE hyphae to decrease. Our findings indicate that altered nutrient limitations shift root associated fungal colonization patterns as well as affect ericaceous root enzyme activity and thereby decomposition potential. Reduction of recalcitrant fungal biomass in melanized DSE may have implications for peatland C sequestration under nutrient addition.

1. Introduction

Peatlands in the Northern hemisphere are often nutrient poor ecosystems characterized by acidic, anoxic, water saturated conditions with considerable nitrogen (N) and phosphorus (P) limitations (Aerts et al., 2001). These conditions and the accumulation of recalcitrant vegetation litter containing high concentrations of phenolic compounds and humic acids inhibitory to microorganisms and vegetation are considered to largely suppress decomposition (Leake and Read, 1990; Painter, 1991; Read et al., 2004; van Breemen, 1995). The challenging conditions in peatlands support a unique diversity of vegetation, with ericaceous species comprising one of the most dominant ground cover groups. Ericaceous shrubs are largely dependent on ericoid mycorrhizal fungi (ERM) to provide access to organic N and P which they provide in exchange for photosynthetic carbon (C) from the host plant (Smith and Read, 2008). The ERM fungi are capable of accessing organic N and P via a large variety of degradative enzymes which act primarily on plant cell wall components (Perotto et al., 2018), demonstrating a potential versatility more comparable to saprotrophs than to other types of mycorrhizae. These ericaceous species are also host to abundant dark septate endophytes (DSE) with extracellular enzyme capabilities potentially capable of improving host nutrient uptake (Mandyam and Jumpponen, 2005, 2014; Upson et al., 2009).

Over the past 150 years atmospheric deposition of N and P in forms easily accessible to plants has been increasing through combustion of fossil fuels and agricultural fertilization (Galloway et al., 2003, 2013; Tipping et al., 2014; Wang et al., 2015). As nutrient limitations are alleviated, ericaceous reliance on ERM fungi may be reduced, potentially altering the symbiont community and leading to the loss of mycorrhizal symbionts. As mycorrhizal symbionts likely play a role in protection against pathogens (Vohník et al., 2016; Weiβ et al., 2016), host species may in turn become more vulnerable to infection. Furthermore, the long-term effects of an altered nutrient balance in peatlands may include reduced nutrient acquisition competitiveness for

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ericaceous species. The ERM fungi are not as efficient decomposers as free-living saprotrophs, which may be naturally kept in check by nutrient limitations and direct competition with ERM fungi (Averill et al., 2014). Reduction of nutrient limitations may free the saprotrophic species' decomposition potential, leading to their dominance and the decline of ERM species and their host plants.

The selective pressure of N deposition on the symbiont community highlights a risk to peatland plant diversity, potentially leading to similar large-scale community shifts as seen in the continental level decline of ectomycorrhizal tree species and the increase in arbuscular tree species described by Averill et al. (2018). Any large-scale changes to peatland microbial and plant communities risk changing the status of peatlands as net C sinks to net sources of greenhouse gas emissions (Andersen et al., 2013), which holds a globally significant potential when considering that peatlands sequester nearly one third of global soil organic carbon C (Gorham, 1991). The risk to C sink potential has been indicated by Larmola et al. (2013), who found that ecosystem C uptake did not increase in a long-term nutrient addition experiment at a nutrient-poor peatland in Canada simulating atmospheric N deposition.

The Whim Bog experimental site, located in the Scottish Borders, was established in 2001 to study the effects of different N forms and P addition on an ombrotrophic peat bog (Sheppard et al., 2004). This site allowed us to study nutrient addition effects on the ericaceous species *Calluna vulgaris* and *Erica tetralix* and their root associated fungi. The primary goals of this study were to characterize the frequency and morphology of mycorrhizal colonization in these ericaceous shrub species, assess ericoid mycorrhizal root enzyme capability related to organic matter degradation, and identify their fungal symbionts under changing nutrient availability. We hypothesized that: (1) the frequency of microscopically observed fungal colonization in ericaceous shrub roots is reduced across both forms of N and NP nutrient addition treatments, reflecting a reduction in reliance on symbionts for nutrient uptake; (2) nutrient addition treatments alter root associated fungal diversity, as determined by morphotypic analysis and ITS sequencing; and (3) the activities of ericoid mycorrhizal root surface enzymes reflect treatment nutrient limitations.

2. Materials and methods

2.1. Study site

The study site, Whim Bog, located in the Scottish Borders, UK (Latitude: 55.76670, Longitude: −3.26667), has undergone nutrient addition treatment since 2001. The four nutrient addition treatments included in this study received annually 6.4 g N m^{-2} either as sodium nitrate ($NaNO_3$) or as ammonium chloride (NH_4Cl), both with and without P and K (as K_2HPO_4). K_2HPO_4 was added at a 1:14 P:N ratio to represent the ratio found in amino acids (See Sheppard et al. (2004) and Levy et al. (2019) for details). The ambient deposition in controls was 0.8 g N m^{-2}. Precipitation collected at the site was mixed with standard solutions to the required treatment concentrations. When adequate precipitation was collected an automated sprayer-system applied the treatment to the plots, simulating natural rainfall. Natural precipitation was not excluded from plots. Plots received 15 years of nutrient addition resulting in a 96 g N m^{-2} cumulative load.

2.2. Site measurements and sampling

In August 2016, plant species composition and abundance for each plot were measured via the point-intercept method using a 0.36 m^2 frame on permanent vegetation quadrats established on site. The frame was placed at a height of ca. 1 m relative to the surface of the plot and a graduated pin was used to measure the frame height and vertical location of each vegetation point for 61 intercepts as described in Larmola et al. (2013). Water table (WT) depth for each plot was measured from holes present after extraction of ingrowth cores used in a separate study, relative to moss surface height.

Triplicate plots per treatment and controls were sampled in November 2016 for *Calluna vulgaris* (L.) Hull and *Erica tetralix* (L.) plant roots from one individual plant per plot. Fine root sections were collected from several points throughout the root system of each sampled plant and stored at 8 °C prior to transport, followed by storage at −20 °C. Each root sample was split into two subsamples: one for microscopy and the other for enzymatic and subsequent molecular analyses.

Surface peat from triplicate plots per treatment and controls was sampled to a depth of 20 cm in each plot and stored at 8 °C prior to transport, followed by storage at −20 °C. After melting, the pH of each sample was measured following homogenization with deionized water at a 1:4 ratio.

2.3. Microscopy

The mycorrhizal status of the ericaceous shrub species *C. vulgaris* and *E. tetralix* was determined via light microscopy and Trypan Blue staining as described in Kiheri et al. (2017). Using the magnified intersections method described by Mcgonigle et al. (1990), with one slide per species from each of three replicate plots per treatment and controls (15 samples per species), using 300 counts per slide, mycorrhizal colonization was quantified according to different morphological categories, described in Table 1. These categories were used to estimate differences between the frequencies of potential ericoid mycorrhizal (ERM) hyphae and typical dark septate endophyte (DSE) hyphae. In our study, only those fungi which were robust in structure, heavily melanized and displaying septa were assessed to be DSE. This was necessary to prevent misclassification of those ERM species which produce melanized and septate hyphae (Vohník and Albrechtová, 2011).

Hereon, putative ericoid mycorrhizal morphotypes are referred to with categories ERM1-4 and dark septate endophytes with DSE1-4 (Table 1). Of the morphotypic categories, only ERM category 1 (intracellular coiling) was interpreted as mycorrhizal colonization frequency. Categories 2–4 were interpreted as potential changes in fungal diversity and function.

2.4. Enzyme assays

The enzymatic activities of root samples from *C. vulgaris* and

Table 1
Microscopic morphological categories of ericaceous root associated fungi.

Hyphal Type	Quantification Category	Morphological Characteristics
	ERM0/DSE0	No fungal presence
Potential ericoid mycorrhizae (ERM)	ERM1	Intracellular coiling
	ERM2	Intracellular hyphae
	ERM3	Colonizing surface hyphae
	ERM4	Extracellular surface hyphae
Dark Septate Endophyte (DSE)	DSE1	Intracellular coiling
	DSE2	Intracellular hyphae
	DSE3	Colonizing surface hyphae
	DSE4	Extracellular surface hyphae

E. tetralix were determined using a multi-enzyme assay described by Pritsch et al. (2011), originally performed for assessing ectomycorrhizal exo-enzyme potential. From triplicate plots of each of the five treatments, rhizomes of both ericaceous species were each sampled for 9 individual ca. 1 cm root pieces, for a total of 270 root samples (n = 5 treatments x 3 plots x 2 species x 9 root samples). To measure eight different hydrolytic and oxidative root surface enzyme potentials we used the method described by Velmala et al. (2014) for fluorescences representing the potential activities of leucine aminopeptidase (EC 3.4.11.1), hemicellulases via β-glucuronidase (EC 3.2.1.31) and β-xylosidase (EC 3.2.1.37), cellulases via cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21), chitinase via N-acetylglucosaminidase (EC 3.2.1.14), and acid phosphatase (EC 3.1.3.2). Samples were incubated at room temperature in the dark and under agitation at 180 rpm on a tabletop shaker, with each root piece in individual wells of 96-well filter plates (30–40 μm mesh size, AcroPrepTM 96 Filter Plate; PALL, Port Washington, NY, USA) in buffers containing enzyme specific 7-amino-4-methylcoumarine (AMC) or 4-methylumbelliferone (MU) substrates. Incubation times for each enzyme followed the protocol of Pritsch et al. (2011). The respective substrates used were Leucine-AMC, MU-xylopyranoside, MU-β-D-glucuronide, MU-cellobiohydrofuran, MU-N-acetyl-β-D-glucosaminide, MU-β-D-glucopyranoside, and MU-phosphate. After incubation, substrate solutions were collected by centrifugation with a 96-well plate adapter at 3200 rpm onto Optiplate-96F reading plates (PerkinElmer, Waltham, MA, USA) containing stop buffer (pH 10). Each substrate's fluorescence was measured using a Victor^3 1420 multilabel plate counter (PerkinElmer, Waltham, MA, USA) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Standard solutions were prepared using aminomethylcoumarin (AMC) and 4-methylumbelliferone (MUF) and used to calculate enzymatic activities from concentrations of released AMC or MUF according to their respective substrates. All standard and enzyme substrate solutions were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Further, laccase (EC 1.10.3.2) activity was used as an indicator of lignin modification activity and was determined by incubation in buffer containing diammonium 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) and colorimetric measurement using a Tecan Infinite M200 PRO Multimode Reader (Tecan Trading AG, Männedorf, Switzerland).

Following enzymatic measurements, the individual root pieces were scanned at high resolution (650 dpi) and their surface area measured using winRHIZO Pro (ver. 2017, Regent Instruments Inc.) software. These surface area values were used to convert enzymatic activity to pmol mm^{-2} min^{-1}.

2.5. Molecular methods and sequencing

From triplicate plots of each of the five treatments, rhizomes of both ericaceous species were each sampled for 9 individual ca. 1 cm root pieces, for a total of 270 root samples (n = 5 treatments x 3 plots x 2 species x 9 root samples). Following enzyme assays and winRHIZO analysis on these samples, a randomized subset of 1 of 3 treatment replicates was taken from the 270 samples for sequencing. These 90 samples, plus four repeated samples, were then directly amplified using a Phire Plant Direct PCR kit's plant leaf protocol (Thermo Fisher Scientific, Waltham, MA, USA) and PCR using the ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) primer pair. Individual root pieces were manually crushed using sterile pestles in provided dilution buffer, in order to release fungal cells both on the root surface and within the root structure, and 1 μl of each mixture was then used as a template in a 20 μl PCR reaction. Cycling conditions for the Direct PCR were: initial denaturation 98 °C 5 min, 40 cycles (98 °C for 5 s, 57 °C for 5 s, 72 °C for 20 s), and final extension 72 °C for 1 min. The Direct PCR products were separated in a 2% agarose gel in 1X TAE buffer at 120V for 2 h and each ITS band excised and purified using a Nucelospin® Gel and PCR Clean-Up kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The purified products were then further amplified using a DreamTaq PCR mastermix (Thermo Fisher Scientific, Waltham, MA, USA) by using 1 μl of Direct PCR product as the template in a 20 μl DreamTaq PCR reaction with the same ITS1F-ITS4 primer pair. The DreamTaq PCR was performed with the following program: initial denaturation 95 °C for 3 min, 35 cycles (95 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min), and final extension 72 °C for 10 min. This was done as a quick method to identify common fungal species in each root sample which could then be linked to their associated root surface enzyme activities. These ITS products were Sanger sequenced using the ITS4 primer (Macrogen Europe, Amsterdam, NL). This produced ITS fragments ranging from 98 to 884 bp in length. This method resulted in the successful sequencing of 60% of (57 out of 94) ITS products (Supplementary File 1), of which 38 sequences were more than 200 bp in length and of high enough quality required for NCBI Gen Bank submission. The sequences are disposed to Gen Bank under the accession numbers **MN059889-MN059927**. See Supplementary File 1 for all 57 FASTA sequences. The high proportion of sequencing failure was likely due to mixed ITS products from multiple fungal species and inhibitory chemistry. Sequence identification was performed using the Unite massBLASTer analysis (Nilsson et al., 2019) on manually trimmed sequences. Analyzed sequences had 86–100% sequence similarity to existing reference or representative sequences within the INSD or Environmental databases and the most likely species hypotheses (SH) were selected as their fungal identities. Functional roles were then assigned to these fungal identities according to the web based FUNGuild bioinformatic tool (Nguyen et al., 2016).

2.6. Statistical analyses

Hereon, α level for statistical significance is defined as $p \leq 0.05$ and indicative as $0.05 < p \leq 0.1$ in all cases, and the term significant specifically indicates statistical significance as $p \leq 0.05$. All analyses were calculated using values from treatment means (n = 3). Differences between treatments, for each host plant separately, were determined by analysis of variance (ANOVA) on logarithmically transformed data followed by pairwise comparisons using the parametric Tukey's HSD and nonparametric Games-Howell post hoc tests, using IBM SPSS Statistics 25. Fungal-Enzyme activity profiles in Fig. 8 were prepared using OriginPro 2018 by assigning fungal identifications to enzyme activities on an individual root piece basis.

The combined data for both host plants' mycorrhizal morphotype categories ERM1-4 and DSE1-4 (Table 1), root enzyme activities, plot level vegetation abundance, surface peat pH, and water table depth were analyzed using correlation analyses. Correlation analyses were performed using rcorr-function with Spearman rank based correlation from package Hmisc v4.1-1 (Harrell et al., 2014) and plotted using corrplot-function from package corrplot v0.84 (Wei and Simko, 2016) in the R programming environment (R Core Team, 2017).

3. Results

3.1. Vegetation, peat pH, and water table

In all four nutrient addition treatments, the dominant ericaceous shrub, *Calluna vulgaris*, tended to decrease in abundance while *Erica tetralix* tended to increase, especially in $NaNO_3$ treatments (Table 2). Nutrient addition treatments showed decreasing trends in the abundance of *Sphagnum*, when compared with controls. The sedge *Eriophorum vaginatum* showed increasing trends in abundance in all treatments except $NaNO_3$, when compared with controls. The high abundance of reported *E. vaginatum* in the $NaNO_3$+PK treatment was largely an effect of one plot where the point-intercept measurements were performed within a large *E. vaginatum* tussock. Furthermore, the different forms of N addition were found to cause opposite changes in peat pH, with $NaNO_3$ increasing pH by ca. 0.2–0.3 units and NH_4Cl decreasing pH by ca. 0.2–0.4 units (Table 2). Effects of treatments on peat pH were found

Table 2
Vegetation abundance (hits per m^2), surface (0–20 cm) peat pH, and treatment water table (WT) depth with ±1 standard deviation, n = 3. Different superscript letters indicate significant differences (P < 0.05) compared with the other treatments. Statistically significant differences were only observed for pH values ($F_{(4,10)} = 6.410$).

	Calluna vulgaris	*Erica tetralix*	*Eriophorum vaginatum*	Other Vasc. spp.	*Sphagnum*	Other Mosses	pH	WT (cm)
Control	590.7 ± 114.6	11.1 ± 16.8	117.5 ± 109.0	5.5 ± 7.3	67.5 ± 59.7	78.7 ± 57.0	4.1 ± 0.0^{ab}	16.0 ± 12.4
$NaNO_3$	435.1 ± 246.2	158.3 ± 97.6	64.8 ± 8.0	15.7 ± 24.8	32.4 ± 56.1	70.3 ± 5.7	**4.4 ± 0.2^{a}**	12.6 ± 9.8
$NaNO_3$ +PK	169.4 ± 140.8	30.5 ± 50.5	570.3 ± 619.6	13.8 ± 10.0	0.0	97.2 ± 38.4	**4.3 ± 0.1^{a}**	8.0 ± 1.4
NH_4Cl	615.7 ± 169.2	52.7 ± 41.9	158.3 ± 89.1	0.0	29.6 ± 17.8	56.4 ± 67.2	3.9 ± 0.1^{ab}	16.0 ± 2.6
NH_4Cl+PK	453.7 ± 247.0	68.5 ± 80.9	314.8 ± 219.6	3.7 ± 6.4	26.8 ± 27.2	68.5 ± 27.8	**3.7 ± 0.1^{b}**	24.0 ± 4.2

to be statistically significant ($F_{(4,10)} = 6.410$), with pairwise comparisons finding statistically significant differences between $NaNO_3$ and NH_4Cl+PK treatments and between $NaNO_3$+PK and NH_4Cl+PK treatments. Mean water table (WT) depth, measured relative to moss surface, was eight cm closer to the moss surface in $NaNO_3$+PK treatments than in control plots, while NH_4Cl treatments affected WT the least. This was likely affected by the loss of moss abundance and subsequent subsidence in $NaNO_3$+PK treatments.

3.2. Root associated fungal morphology and frequency

The mean proportion of root intersects which were microscopically quantified as presenting fungal colonization increased from 78% to 83% in control plots for *C. vulgaris* and *E. tetralix* roots, respectively, to 89% and 91% in nutrient addition plots, respectively. Of these root intersections which contained fungal structures, both ericaceous host species showed trends of increasing ERM hyphal frequency and decreasing DSE hyphal frequency when under nutrient addition (Fig. 1). Nutrient addition increased mean ERM hyphal frequency in *C. vulgaris* and *E. tetralix* roots by 31% and 16%, respectively. However, mean DSE hyphal frequency decreased by 30% in *C. vulgaris* roots and 22% in *E. tetralix* roots. Though these trends are considerable, they were not found to be statistically significant due to high natural variation between samples.

Intracellular hyphal frequency (ERM2, Table 1) of *C. vulgaris* roots significantly ($F_{(4,10)} = 11.406$) increased by ca. two-fold under nutrient addition when comparing controls to $NaNO_3$+PK, NH_4Cl, and NH_4Cl+PK, as well as increasing by approximately half under $NaNO_3$ addition, which was a statistically indicative change (Fig. 2). *C. vulgaris* roots showed no statistically significant increases in cells containing typical ERM intracellular coiling (ERM1), root surface colonizing hyphae (ERM3), or extracellular surface hyphae (ERM4) under nutrient

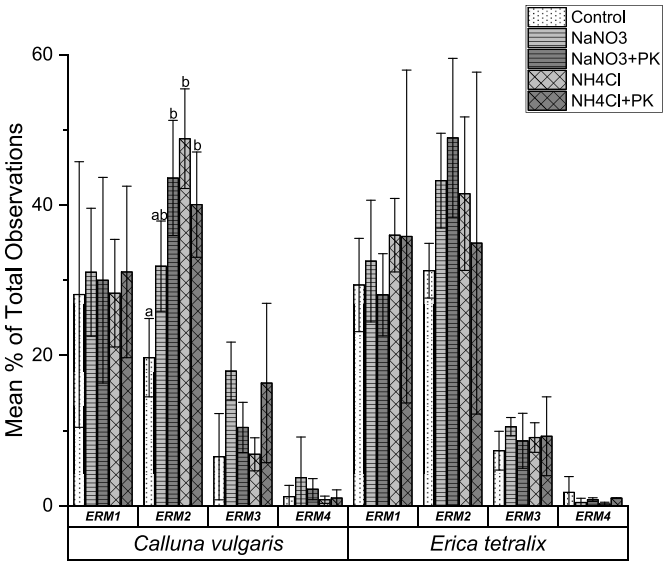

Fig. 2. Microscopically observed frequencies of potential ericoid mycorrhizal morphotypes (ERM1-4) in *C. vulgaris* and *E. tetralix* roots. Error bars indicate ±1 standard deviation, n = 3. Different superscript letters indicate significant differences (P < 0.05) compared with the other treatments.

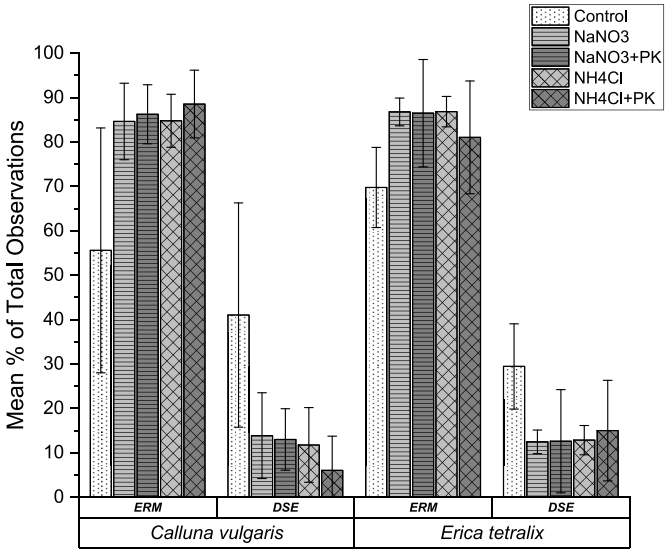

Fig. 1. Total observed ericoid mycorrhizal (ERM) and dark septate endophyte (DSE) occurrence in *C. vulgaris* and *E. tetralix* roots as determined by light microscopy and the magnified intersections method. Error bars indicate ±1 standard deviation, n = 3.

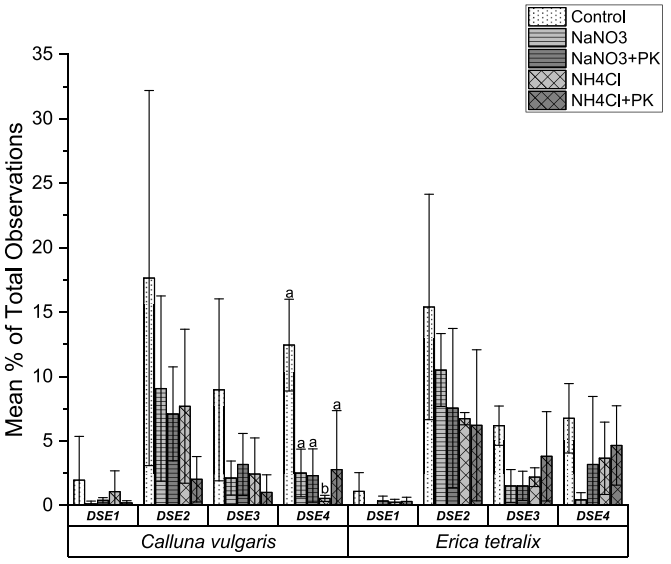

Fig. 3. Microscopically observed frequencies of dark septate endophyte morphotypes (DSE1-4) in *C. vulgaris* and *E. tetralix* roots. Error bars indicate ±1 standard deviation, n = 3. Different superscript letters indicate significant differences (P < 0.05) compared with the other treatments.

addition. *C. vulgaris* DSE extracellular hyphal frequency (DSE4) decreased significantly under NH_4Cl addition to less than 10% that of controls ($F_{(4,10)} = 4.654$) (Fig. 3). The same analysis for *E. tetralix* roots showed similar trends of increasing ERM morphotype frequencies under nutrient addition (Fig. 2), while DSE morphotype frequencies decreased (Fig. 3). Interestingly, a statistically indicative positive correlation was found between *E. tetralix* root surface DSE colonization frequency (DSE3) and plot *Sphagnum* abundance (r = 0.58) (Fig. 5).

3.3. Root enzymatic activity

The different forms of N addition, NH_4^+ and NO_3^-, induced variable and opposing effects on C and N acquiring enzymes on the two ericaceous shrubs' root surfaces, when compared to controls (Figs. 6 and 7). Treatment with $NaNO_3$ tended to reduce all *C. vulgaris* root enzymatic activities except acid phosphatase, suggesting that *C. vulgaris* or its root associated fungi are more sensitive to $NaNO_3$ than *E. tetralix*, which did not show this effect. In *C. vulgaris* roots under NH_4Cl addition, the activities of the C acquiring enzymes β-xylosidase and β-glucosidase did not change compared to controls, while activity of β-glucuronidase tended to increase two-fold and N-acetylglucosaminidase tended to decrease by nearly half. In contrast, the effects of both forms of N on *E. tetralix* roots were similar for all C and N acquiring enzymes, while leucine aminopeptidase activity tended to be suppressed under NH_4Cl addition to less than 25% of controls and N-acetylglucosaminidase decreased by less than half, compared to controls. Laccase activity was not detected in any samples.

Compared to control plots, addition of both forms of N alone tended to induce an approximately 25% increase in acid phosphatase activity in both plant species (Figs. 6 and 7). In contrast, treatments with additional PK reduced acid phosphatase activity to 1/3rd of controls in both ericaceous species (Fig. 7). In *C. vulgaris* roots, the effect of nutrient additions on acid phosphatase activity was statistically significant ($F_{(4,10)} = 8.163$), with significant differences between $NaNO_3$ and $NaNO_3$+PK and NH_4Cl+PK treatments, as well as between NH_4Cl and $NaNO_3$+PK and NH_4Cl+PK treatments. In *E. tetralix* roots the effect of nutrient addition on acid phosphatase activity was significant ($F_{(4,10)} = 11.400$), with significant differences between control and NH_4Cl+PK treatments, between $NaNO_3$ and $NaNO_3$+PK and NH_4Cl+PK treatments, and also between NH_4Cl and $NaNO_3$+PK and NH_4Cl+PK treatments.

Interestingly, in both ericaceous species the NPK treatments induced higher, although statistically non-significant, activities in many C and N acquiring enzymes compared to N alone (Figs. 6 and 7). The exceptions to this were the suppression of β-xylosidase and β-glucuronidase activities in *C. vulgaris* roots under NH_4Cl+PK addition. Additionally, there was a significant positive correlation between *C. vulgaris* ERM intracellular hyphal frequency (ERM2) and β-glucuronidase activity (r = 0.66) and a statistically indicative positive correlation with β-glucosidase activity (r = 0.50) (Fig. 4).

3.4. Ericaceous root associated fungi

Sanger sequencing of Direct PCR ITS amplicons from individual root samples from both ericaceous species revealed several confirmed and putative ERM/DSE fungal species, as well as a range of possible endophytes, saprotrophs, and pathogens (Table 3). Fungal sequence identifications based on Species Hypotheses (SH) ranged from 86% to 100% matches with reference sequences. Identifications of ascomycete fungi likely inhabiting these ericaceous roots as ERM symbionts included *Hyaloscypha hepaticicola* and *Hyaloscypha* sp., (names updated from

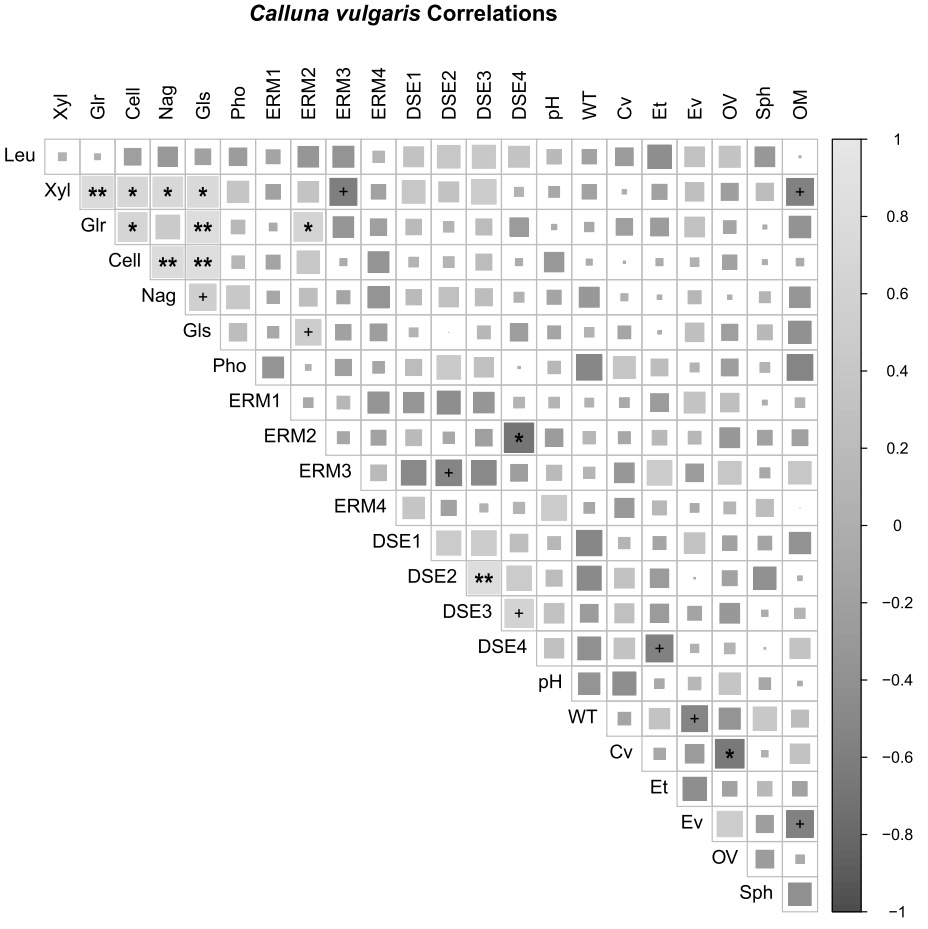

Fig. 4. Correlation analysis of *Calluna vulgaris* root enzyme activities (Leu: leucine aminopeptidase, Glr: β-glucuronidase, Xyl: β-xylosidase, Cell: cellobiohydrolase, Gls: β-glucosidase, Nag: N-acetylglucosaminidase, Pho: acid phosphatase), root associated fungal morphotype categories ERM1-4 and DSE1-4 (Table 1), surface peat (0–20 cm) pH, plot water table depth (WT), *C. vulgaris* abundance (Cv), *Erica tetralix* abundance (Et), *Eriophorum vaginatum* abundance (Ev), other vascular spp. abundance (OV), *Sphagnum* abundance (Sph), and other moss spp. abundance (OM). Statistically significant and indicative correlation values are indicated by asterisks (** for $p < 0.01$, * for $p < 0.05$, and + for $p < 0.1$) values, with a greyscale ranging from lighter shades indicating positive correlation coefficients to darker indicating negative correlation coefficients, n = 15.

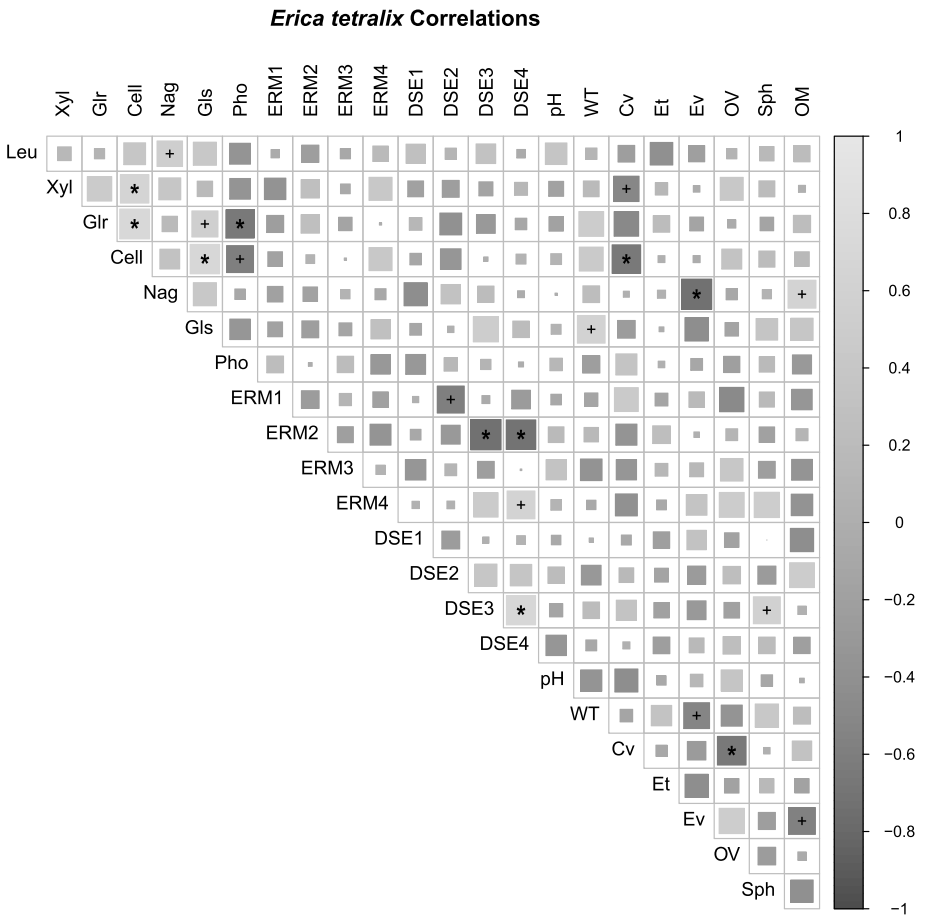

Fig. 5. Correlation analysis of *Erica tetralix* root enzyme activities (Leu: leucine aminopeptidase, Glr: β-glucuronidase, Xyl: β-xylosidase, Cell: cellobiohydrolase, Gls: β-glucosidase, Nag: N-acetylglucosaminidase, Pho: acid phosphatase), root associated fungal morphotype categories ERM1-4 and DSE1-4 (Table 1), surface peat (0–20 cm) pH, plot water table depth (WT), *Calluna vulgaris* abundance (Cv), *E. tetralix* abundance (Et), *Eriophorum vaginatum* abundance (Ev), other vascular spp. abundance (OV), *Sphagnum* abundance (Sph), and other moss spp. abundance (OM). Statistically significant and indicative correlation values are indicated by asterisks (* for $p < 0.05$ and + for $p < 0.1$) values, with a greyscale ranging from lighter shades indicating positive correlation coefficients to darker indicating negative correlation coefficients, n = 15.

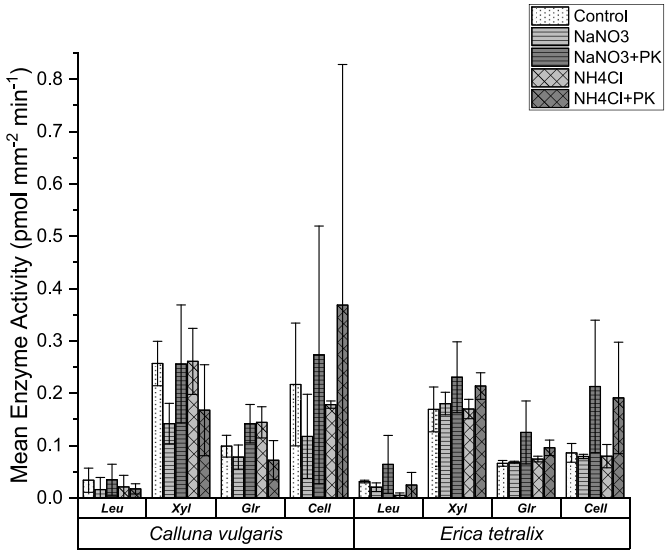

Fig. 6. *C. vulgaris* and *E. tetralix* root surface enzyme activities according to treatment means (Leu: leucine aminopeptidase, Xyl: β-xylosidase, Glr: β-glucuronidase, Cell: cellobiohydrolase). Error bars indicate ±1 standard deviation, n = 3.

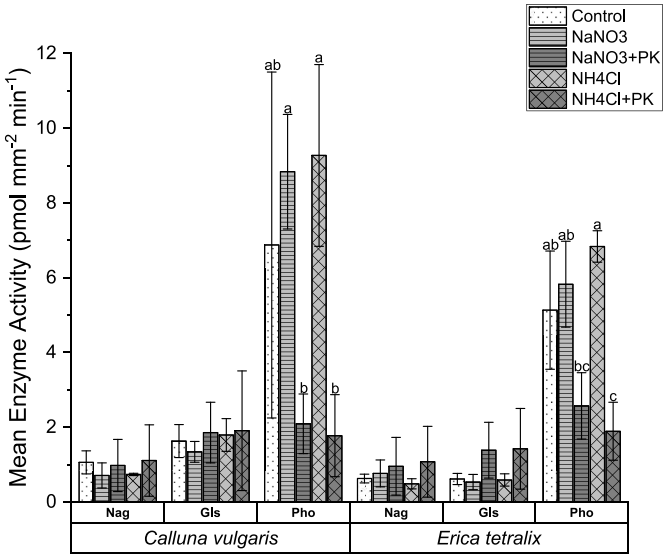

Fig. 7. *C. vulgaris* and *E. tetralix* root surface enzyme activities according to treatment means (Nag: N-acetylglucosaminidase, Gls: β-glucosidase, Pho: acid phosphatase). Error bars indicate ±1 standard deviation, n = 3. Letters above error bars indicate statistically significant differences.

Rhizoscyphus ericae and *Meliniomyces* sp., respectively, according to Fehrer et al. (2019)). Other members of the Leotiomycetes were also identified, including unidentified Helotiales, unidentified *Hyaloscyphaceae*, *Phialocephala sphaeroides*, and *Pseudogymnoascus* sp. Furthermore, identified basidiomycetes which are capable of the ERM lifestyle included members of the family *Serendipitaceae* (syn. clade B Sebacinales) and its member genus *Serendipita*. Interestingly, members of the Helotiales were found only in roots of both hosts from control, NH_4Cl or NH_4Cl+PK treatments while unidentified Pezizales members were only detected in $NaNO_3$+PK treatments. Other ericaceous root associated

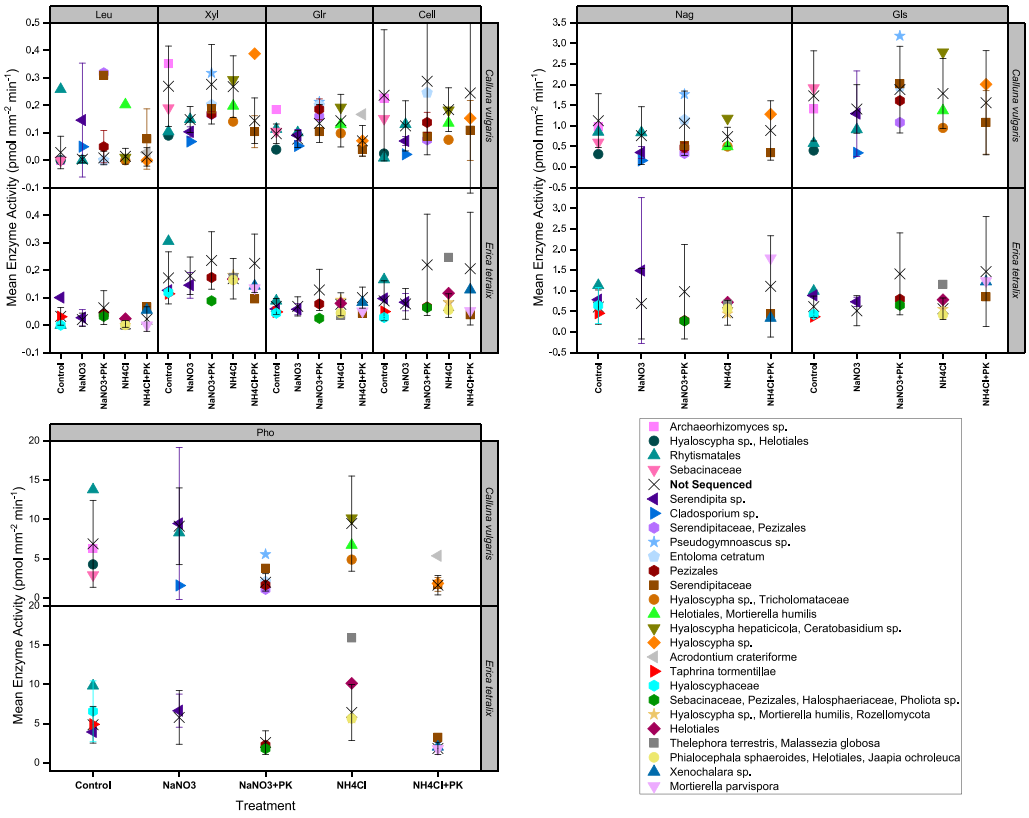

Fig. 8. Ericaceous root enzyme activities (Leu: leucine aminopeptidase, Glr: β-glucuronidase, Xyl: β-xylosidase, Cell: cellobiohydrolase, Gls: β-glucosidase, Nag: N-acetylglucosaminidase, Pho: acid phosphatase) for individual root pieces with identified fungal sequences, as well as the mean activities for root samples without sequencing (Black X Symbol, 20<n<25, per treatment). Error bars indicate ±1 standard deviation. Not shown; *Acrodontium crateriforme*: Xyl = 0.61, Nag = 7.5, (*C. vulgaris*, NH_4Cl+PK). *Pseudogymnoascus* sp.: Cell = 0.87, (*C. vulgaris*, $NaNO_3$+PK).

fungi which were possibly living saprotrophic or pathotrophic lifestyles are presented in Table 3.

3.5. Ericaceous root enzyme activity profiles

When fungal sequence identifications were linked to the enzymatic activities of their respective root samples, potential species related enzyme activity patterns emerged (Table 3, Fig. 8). *C. vulgaris* root samples from $NaNO_3$, $NaNO_3$+PK, and NH_4Cl+PK treatments and hosting *Serendipitaceae* (Clade-B Sebacinales) or *Serendipita* sp. produced the highest detected leucine aminopeptidase activities. A *C. vulgaris* root sample in the NH_4Cl+PK treatment and hosting *Hyaloscypha* sp. was highly active in β-xylosidase, N-acetylglucosaminidase, and β-glucosidase, while samples hosting *Hyaloscypha* sp. in control and NH_4Cl treatments were ca. 50–75% less active for the same enzymes. Additionally, a *C. vulgaris* root sample in the NH_4Cl treatment simultaneously hosting *Hyaloscypha hepaticicola* and *Ceratobasidium* sp. was one of the most active samples, across all enzymes.

Interestingly, a root sample from *C. vulgaris* in the $NaNO_3$+PK treatment was linked to a fungal identification of *Pseudogymnoascus* sp., which produced some of the highest activities for all enzymes in this treatment, except leucine aminopeptidase. A sample of *C. vulgaris* root from the NH4Cl+PK treatment which hosted the species *Acrodontium crateriforme* indicated activities of cellobiohydrolase, N-acetylglucosaminidase, and β-glucosidase several times higher than any other samples measured. In the control treatment, *C. vulgaris* root samples hosting a member of the Rhytismatales showed the highest activity for leucine aminopeptidase and acid phosphatase while *E. tetralix* samples hosting Rhytismatales showed the highest activities for all enzymes in that treatment, except leucine aminopeptidase.

4. Discussion

The increases in overall fungal colonization for both *Calluna vulgaris* and *Erica tetralix* under nutrient addition were unexpected and the unchanged frequency of ERM intracellular coiling showed that long-term N and NPK addition did not reduce mycorrhizal colonization. This suggests that the ericaceous host plants are unable to restrict fungal colonization of their roots, despite access to excess inorganic N and P. Alternatively, unchanged mycorrhizal colonization rates may indicate that the fungi provide benefits to the plant beyond N and P.

The observed reduction of *Sphagnum* abundance and the decreased frequency of DSE associated with ericaceous roots may be linked. DSE fungi have been shown to be a common occurrence in submerged aquatic plants (Kohout et al., 2012) and are capable of propagating and existing as saprobes of moss gametophytes (Day and Currah, 2011). This capability and their prevalence in aquatic plants suggest that DSE fungi are well adapted to periodic waterlogged conditions in peatlands, perhaps providing a niche during the annual senescence of their ericaceous hosts. A similar relationship between ERM fungi and liverworts, common species in peatlands, may also provide a niche during host senescence (Kowal et al., 2018, 2015). As the loss of *Sphagnum* species is one of the most obvious effects of nutrient addition treatments (Bubier et al., 2007; Levy et al., 2019), this may remove an important ecosystem niche for DSE fungi. Our finding of positive correlation between *E. tetralix* root surface DSE colonization frequency and *Sphagnum* abundance supports this possibility.

The significantly increased frequencies of ERM hyphal morphotypes and decreased DSE hyphal morphotypes in ericaceous roots strongly suggests that long-term nutrient addition resulted in an altered fungal community. Furthermore, the different forms of N addition may have had selective effects on the fungal community as we identified different root associated Helotiales members only in controls, NH_4Cl or NH_4Cl+PK treatments and members of the Pezizales only in $NaNO_3$+PK treatments. The Helotiales may prefer NH_4^+ as a substrate compared to NO_3^-, as experimental evidence has shown for *Hyaloscypha hepaticicola* (Cairney et al., 2000). The presence of the Pezizales in only $NaNO_3$+PK treatments may imply they prefer this N source, though their

Table 3
Fungal identifications from Sanger sequencing of Direct PCR ITS amplicons from *C. vulgaris* and *E. tetralix* roots. % ID values indicate range of similarity with reference sequences according to Unite database. Function assignment according to FUNGuild analysis. Sequences are from 38 samples with ITS amplicons >200 bp as required by Unite Gen Bank, see Supplementary File 1 for all 57 FASTA sequences. Sources of sequences listed by treatments and host species indicated with Cv and Et, numbers in brackets indicate the number of replicate plots with the same sequence, subscripts c = Control, 1 = $NaNO_3$, 2 = $NaNO_3$+PK, 3 = NH_4Cl, 4 = NH_4Cl + PK.

Phylum	Class	Order	Family	Species	% ID	Function	Reference Seq(s)|SH	Source
Ascomycota	Archaeorhizomycetes	Archaeorhizomycetales	*Archaeorhizomycetaceae*	***Archaeorhizomyces* sp.**	97.58	Sapro	KT768305|SH180923.07FU	Cv_c
	Dothideomycetes	Capnodiales	*Mycosphaerellaceae*	***Acrodontium crateriforme***	95.16	Patho-Sapro	KX287271|SH214154.07FU	Cv_4
			Cladosporiaceae	***Cladosporium* sp.**	99.77	Patho-Sapro-Symbio	KX459429|SH212842.07FU	Cv_1
	Leotiomycetes	**Helotiales**	***Hyaloscyphaceae***	***Hyaloscypha hepaticicola***[a]	99.07	Patho-Sapro-Symbio	FM172802|SH181107.07FU	Cv_3
				***Hyaloscypha* sp.**[a]	99.18–99.59	Sapro-Symbio	FM997935|SH025067.07FU DQ309217|SH214267.07FU	Cv_c; Cv/Et_3; Cv_4
				Unidentified	99.79	Sapro	HF947840|SH004619.07FU	Et_c
			Vibrisseaceae	***Phialocephala sphaeroides***	91.06	Symbio	KC480051|SH204990.07FU	Et_3
			Unidentified	Unidentified	88.89–99.61	–	HF947859|SH218310.07FU AF252840|SH211416.07FU HF947861|SH197071.07FU AY627806|SH201639.07FU	Cv_c; $Cv/Et(2)_3$
		Rhytismatales	Unidentified	Unidentified	92.00–95.71	–	AF149078|SH183994.07FU DQ309240|SH143881.07FU	Cv/Et_c; Cv_1
		Thelebolales	*Pseudeurotiaceae*	***Pseudogymnoascus* sp.**	93.89	Patho-Sapro-Symbio	KP902680|SH183329.07FU	Cv_2
	Pezizomycetes	**Pezizales**	Unidentified	Unidentified	95.94–100	–	JQ347011|SH203769.07FU	$Cv(2)/Et(2)_2$
	Sordariomycetes	Microascales	***Halosphaeriaceae***	Unidentified	94.64	Sapro	FJ524322|SH211311.07FU	Et_2
	Taphrinomycetes	Taphrinales	*Taphrinaceae*	***Taphrina tormentillae***	99.14	Patho	KX516468|SH200748.07FU	Et_c
	incertae sedis	incertae sedis	incertae sedis	***Xenochalara* sp.**	96.04	Sapro	HM230882 |SH202721.07FU	Et_4
Basidiomycota	Agaricomycetes	Agaricales	*Entolomataceae*	***Entoloma cetratum***	97.14	Patho-Sapro-Symbio	KC898450|SH185814.07FU	Cv_2
			Tricholomataceae	Unidentified	93.82	Patho-Symbio	KY701558|NA	Cv_3
			Strophariaceae	***Pholiota* sp.**	86.47	Sapro	HQ533029|SH219745.07FU	Et_2
		Cantharellales	*Ceratobasidiaceae*	***Ceratobasidium* sp.**	99.49	Patho-Sapro-Symbio	JN569114|SH220624.07FU	Cv_3
		Jaapiales	*Vibrisseaceae*	***Jaapia ochroleuca***	99.31	Sapro	UDB031153 |SH190037.07FU	Et_3
		Sebacinales	***Serendipitaceae* (Clade B Sebacinales)**	***Serendipita* sp.**	98.25–100	Symbio	GQ907110|SH003898.07FU HF947895|SH201953.07FU DQ309211|SH179088.07FU DQ309149|SH180008.07FU	Et_c; $Cv(2)_1$; $Et(2)_3$
				Unidentified	94.35–100	–	HF947869/HF947915 /DQ309208 |SH179085.07FU	$Cv(2)_2$; Cv/Et_4
			Sebacinaceae	Unidentified	99.66	Symbio	HQ154421|SH199330.07FU	Cv_c;Et_2
		Thelephorales	*Thelephoraceae*	***Thelephora terrestris***	98.72	Symbio	KX438350|SH184510.07FU	Et_3
Mortierellomycota	Mortierellomycetes	Mortierellales	*Mortierellaceae*	***Mortierella humilis***	100	Sapro-Symbio	KM504403|SH196779.07FU	Cv/Et_3
				Mortierella parvispora	99.83	Sapro-Symbio	FN565295|SH193938.07FU	Et_4
Rozellomycota	unidentified	Unidentified	Unidentified	Unidentified	98.23	–	KF297176|SH204524.07FU	Et_3

[a] Sequences identified as *Rhizoscyphus ericae* and *Meliniomyces* sp. updated to *Hyaloscypha hepaticicola and Hyaloscypha sp*, respectively, according to Fehrer et al. (2019).

mycorrhizal status is unclear it has been suggested for some families by Hobbie et al. (2001).

Sequence identification of ITS amplicons from root samples of both ericaceous species revealed several ERM and DSE species commonly found to associate with ericaceous roots, as well as common peatland saprotrophs and pathogens (Sietiö et al., 2018; Thormann, 2006; Thormann and Rice, 2007). Both ericaceous species shared several family and genus level groups, indicating a common symbiont community among the ericaceous hosts. This is in line with the findings of Kjøller et al. (2010) who showed that several ericaceous species in a subarctic mire shared fungal communities when in close proximity. While ERM symbionts are generally ascomycetes, recent studies have found that the basidiomycete fungi *Serendipitaceae* (Clade-B Sebacinales) are common in ericaceous roots and capable of forming mycorrhizal structures (Brundrett and Tedersoo, 2018; Vohník et al., 2016; Weiß et al., 2016), and potentially capable of utilizing photosynthetic C (Sietiö et al., 2018). Our identifications of members of the *Serendipitaceae* and *Serendipita* sp. in both ericaceous hosts support their likely role as root symbionts. Interestingly, in *C. vulgaris* roots from the $NaNO_3$+PK treatment we found a potential ERM symbiont, *Pseudogymnoascus* sp., which is a genus that may form ERM associations, as shown between *Pseudogymnoascus roseus* and *Vaccinium angustifolium* (Dalpé, 1989).

Our findings on the varying effects of N addition on root surface enzymatic activities in the two ericaceous species, *C. vulgaris* and *E. tetralix*, indicate that they may have functionally different root symbionts and decomposition potentials. In NPK treatments both ericaceous plants displayed highly suppressed acid phosphatase activity as the roots and root associated fungi did not need to access organic P sources. Conversely, N treatments increased acid phosphatase activity, reflecting the colimitation of N and P found in peatlands (Pinsonneault et al., 2016; Wang et al., 2015) and confirming that ericoid mycorrhizal root enzymatic activities reflect nutrient limitations. Almost every enzyme activity increased with additional P compared to either form of N alone, while $NaNO_3$ addition was found to generally decrease enzyme activities for *C. vulgaris*. Additionally, both forms of N addition led to reductions in chitinase activity, which is similar to the findings of Bragazza et al. (2006), who suggested that this indicates an alleviation of N limitation.

In *C. vulgaris* roots β-xylosidase, β-glucuronidase, and β-glucosidase activities under NH_4Cl addition were comparable to or higher than their activities in controls and $NaNO_3$+PK treatments, suggesting that NH_4Cl promotes overall decomposition activity, as these enzymes primarily degrade plant cell wall components (Dunn et al., 2014). This may reflect a reduction in N limitation for the *C. vulgaris* mycorrhizal symbiont identified as *Hyaloscypha hepaticicola*, as experimental *in vitro* data has shown that this species may preferentially utilize NH_4^+ as a source of N compared to organic sources (Cairney et al., 2000). Additionally, this is supported by the colonization morphotype data showing that NH_4Cl addition induced the largest significant increase in intracellular ERM hyphae in *C. vulgaris*, implying increases in fungal biomass and therefore higher decomposition potential. Furthermore, the sequence identification of *H. hepaticicola* in a *C. vulgaris* root sample from the NH_4Cl treatment was linked to relatively higher enzymatic activities compared to other root samples in the same treatment. *Calluna vulgaris* roots hosting *Hyaloscypha* sp. displayed higher enzymatic activity under nutrient addition than in control conditions, indicating a response to increased N or NPK availability.

Our results demonstrate that it is necessary for studies of mycorrhizal fungi to include measurements of enzyme activities in natural conditions in order to more precisely estimate their roles in nutrient cycles. Though there is extensive research on the enzymatic activity of mycorrhizal fungi in sterile systems, few studies have measured the activity of mycorrhizal roots in their natural environment. While these *in vitro* enzyme activities of mycorrhizal fungi are often interpreted as their natural activities, work by Timonen and Sen (1998) showed that enzyme expression levels in *Pinus sylvestris* mycorrhizal fungi are locally regulated in the mycorrhizosphere, highlighting the variability in fungal enzyme expression which is not apparent from *in vitro* studies.

We observed that long-term nutrient addition resulted in a reduction in *C. vulgaris* abundance, potentially due to a reduction in competitive fitness, leaving an ecosystem gap that was rapidly occupied by other fast-growing species such as the non-mycorrhizal sedge *Eriophorum vaginatum*, which is not reliant on symbionts for organic N uptake (Chapin et al., 1993). The suppressive nature of $NaNO_3$ on *C. vulgaris* root enzymatic activities, compared to *E. tetralix*, suggests that *C. vulgaris* and its symbionts are more sensitive to $NaNO_3$ and its effects on peat properties, such as pH. This is also reflected in the vegetation abundance data for $NaNO_3$ where *C. vulgaris* abundance is reduced while *E. tetralix* abundance increases. This sensitivity to $NaNO_3$ may put *C. vulgaris* at a competitive disadvantage to other ericaceous species during NO_3^- deposition.

Our findings of the cumulative effects of nutrient addition treatments at Whim Bog on abundances of peatland vegetation are similar to those detailed in Levy et al. (2019) which describes the decline of several plant species and the increase of *E. vaginatum* as the major effects over the entire timespan of the experimental site. The loss of *Sphagnum* may also directly reduce the ability of ericaceous species to uptake nutrients, as the upper moss layer is heavily inhabited by ericaceous roots, forming a thick layer which receives nutrients from litter and the atmosphere before it reaches the lower layers (Read et al., 2004). As the living *Sphagnum* layer is lost and forms bare, decaying peat, it collapses and becomes more submerged and anoxic, becoming an environment that ericaceous roots are less likely to inhabit. This loss of aerobic substrate for ericaceous species to inhabit and uptake nutrients from may be an underlying cause of the observed reduction in *C. vulgaris* abundance. The loss of the moss layer may also lead to the subsidence of peat, as has been observed by Juutinen et al. (2018) to be a result of nutrient addition at another long-term nutrient addition experiment, Mer Bleue Bog, located in Ontario, Canada. This subsidence was indicated by our water table depth measurements, as they were made relative to the moss surface and the treatments with the highest water table values, $NaNO_3$ and $NaNO_3$+PK, also showed the largest reductions in moss abundance. Furthermore, the effects of the treatments on peat pH should not be overlooked as a significant source of variation. Long-term alteration of pH is likely directly linked to the observed differences in fungal colonization patterns as well as root enzymatic activity.

Loss of ericaceous vegetation and *Sphagnum* are key examples of the risks posed by anthropogenic N and P deposition. Current research has lacked a clear picture of how ericaceous root associated fungi, ERM and DSE, are involved in these processes. Our findings indicate that altered nutrient limitations shift root associated fungal diversity and morphology, with direct effects on enzyme activity and thereby decomposition potential. The losses of *C. vulgaris* and *Sphagnum* due to nutrient addition and the subsequent reduction in DSE colonization rates may have additional consequences. Dark septate endophytes are by nature heavily melanized and may contribute a significant source of recalcitrant C (Fernandez and Koide, 2014). Subsequently, the potential loss of recalcitrant fungal biomass may lead to lower peatland C sequestration. As suggested by Averill et al. (2014) and Orwin et al. (2011), ERM symbionts may be responsible for limiting the decomposition potential of free-living saprotrophs and the microbial community by increasing N and P limitation in soil. Addition of inorganic N and P may upset this limitation, leading to increased decomposition which releases C limitation for the more efficient saprotrophs, which in turn outcompete the mycorrhizal fungi, thereby limiting ericaceous nutrient access. Further research is necessary to determine the potential long-term risks of N and P deposition and the role of peatland mycorrhizal fungi in C sequestration.

4.1. Conclusions

The total frequency of fungal colonization at Whim Bog, Scotland, did not decrease under nutrient addition treatments but instead tended

to increase by ca. 10% in both *Calluna vulgaris* and *Erica tetralix*, refuting our hypothesis (1) which expected a reduction in fungal colonization rates. The considerable increase in ERM hyphal frequency (ca. 20–30%) in both host species was accompanied by a significant suppression of DSE hyphal frequency (ca. 20–30%) under nutrient addition, indicating a strong treatment effect on the root symbiont community. The altered fungal morphotype frequencies and identified fungal species agree with our hypothesis (2) of mycorrhizal diversity indicating nutrient addition effects and suggests that altered nutrient availability is a selective pressure upon the root associated fungal community. The enzymatic activities of both ericaceous shrub roots and their associated fungi strongly support our hypothesis (3) of mycorrhizal root enzyme activities reflecting nutrient limitations.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2020.107833.

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