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Fungal endophytes of roots can exhibit host-specificity through overwintering

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Decaying roots may play a role in endophytic fungal survival through an unfavourable season. We investigated root fungal endophytic communities of *Avenella flexuosa* and *Lysimachia europaea* prior to and after a simulated winter. To assess the viability of the endophytic fungi, we used a culture-based approach. We found that the total number of root fungal isolates, the number of isolate morphotypes and the isolate morphotype communities remained host-species-specific through overwintering. Both the total number of fungal isolates and the number of isolate morphotypes were higher in *Avenella* than in *Lysimachia* both prior to and after the simulated winter. The winter simulation also increased both the total number of isolates and the number of isolate morphotypes in *Avenella*, whereas in *Lysimachia*, the simulated winter had no effect. The root fungal isolate morphotype community was more heterogeneous in *Avenella* than in *Lysimachia*. An isolate morphotype identified as *Phialocephala fortinii*, a dark septate endophyte, dominated the root culturable fungal community in *Lysimachia*. Our results suggest that the endophytic fungi of *Avenella* and *Lysimachia* have potential to survive in the decaying root fragments and that the fungal community remains host-species-specific until the next growing season.

Keywords: culturing methods, dark septate endophytes, *Phialocephala fortinii*, seasonality, symbiosis.

Plant roots are closely associated with fungi. The root-associated fungal communities represent different lifestyles, including mycorrhizal, saprotrophic, pathogenic, and endophytic fungi (Smith & Read 2008, Rodriquez et al. 2009, Porras-Alfaro & Bayman 2011). Endophytes are microbes that live asymptomatically inside plant tissues (Hardoim et al. 2015). In grasses, the Clavicipitacean fungal endophytes of class I inhabit the plant systematically and are vertically transmitted via seeds between host plants (Rodriguez et al. 2009). For other fungal endophytes belonging to classes II and IV and representing root-associated lifestyles, horizontal transmission, i.e., individual colonization of each host plant generation, is more common (Rodriguez et al. 2009, Stroheker et al. 2024). The ability to grow and colonize a new host generation after an unfavourable season is likely important for root endophytic fungi.

Root endophytic fungi can exhibit a degree of host specificity (Gange et al. 2007, Kernaghan & Patriquin 2011, Tejesvi et al. 2013, Botnen et al. 2020). Apart from the mycorrhizal fungi that are more tightly associated with certain plant families (for example, ericoid mycorrhizal fungi), root fungal endophytes belonging to dark septate endophytes (DSE) are commonly found in most plant families worldwide, suggesting they are less host-specific (Jumpponen & Trappe 1998, Smith & Read 2008). However, also the DSE-type fungi colonizing neighbouring herbaceous plants have been found to exhibit a degree of host-preference (Tejesvi et al. 2013). Because mycorrhizal and other fungal associations affect plant growth and performance (Smith & Read 2008, Rodriquez et al. 2009), studying host preference of root endophytic fungi could contribute to the understanding of plant-plant and plant-fungal relationships in general terms.

A number of foliar endophytes inhabiting plant leaves (Gange et al. 2007, Eschen et al. 2010) are also found in the soil (Domsch et al. 2007), which raises questions about their life-cycle between the soil, roots and shoots. Previous studies have indicated that fungal endophytic communities can vary between plant species, being affected by the soil nutrient status and the level of arbuscular mycorrhizal (AM) colonization (Gange et al. 2007, Eschen et al. 2010). Foliar fungal endophyte communities have been found to have between-season cycles (e.g., Petrini et al.1992, Wearn et al. 2012, Taniguchi et al. 2023). In addition to studies of within-season root fungal endophyte phenology (Ruotsalainen et al.

2002, Knapp et al. 2012) there are only few field studies on between-season variation of root fungal endophytic communities (Li et al. 2005, Wearn et al. 2012, Taniguchi et al. 2023). For arbuscular mycorrhizal fungi, the decaying roots and root fragments are known to carry fungal hyphae and spores and act as a source of fungal inoculum, i.e. new fungal growth (Klironomos & Hart 2002, Pepe et al. 2018). However, the role of decaying host root fragments in the between-season survival of root fungal endophytic communities is less well known.

A crucial step in understanding how communities of fungal endophytes act and interact within their hosts is the isolation and identification of the specific members forming the association. The existence of isolates enables viability assessment, recolonization studies, and manipulation of the interaction for functional studies in plants (Finkel et al. 2017). We have previously reported contrasting root fungal isolate communities of neighbouring plant species *Avenella flexuosa* and *Lysimachia europaea* roots collected at the end of the growing season (Tejesvi et al. 2013). Here, as a continuation of that study, we aim to elucidate changes in the fungal communities of detached roots of the two plant species prior to and after a simulated winter period in controlled conditions. The root fungal communities were studied by isolating viable fungi from surface-sterilized roots into living cultures prior to and after a simulated winter.

Materials and methods

Sampling and preprocessing of roots

We sampled roots of *Avenella flexuosa* (L.) Drejer (Poaceae) and *Lysimachia europaea* (L.) U. Manns & Anderb. (Primulaceae) from two northern boreal forest sites located near the city of Oulu (65°01' N, 25°30' E), Northern Finland in late autumn. Both sampling sites represent *Pinus sylvestris* L. and *Betula pubescens* Ehrh. dominated mixed forest with the field layer characterized by *A. flexuosa*, *Vaccinium myrtillus* L. and *V. vitis-idaea* L. Sampling was carried out by using a soil borer (15 cm in diameter). The soil cores were kept in +4 °C before further processing. The roots of both target plants were cleaned from the soil cores under a dissection microscope. Detached root samples were each divided into three subsamples: one part was cleaned and placed in 70 % ethanol for a microscopic root colonization analysis, one part was directly plated to obtain fungal isolates, and the third part was used for the simulated winter experiment, hereafter winter incubation. Root fungal colonization % and fungal isolate communities of field root subsamples have been reported in Tejesvi et al. (2013).

Isolating and morphotyping root-associated fungi

Isolation and morphotyping of the fungal isolates were carried out prior to and after the winter incubation as follows: Fresh roots were surface-sterilized with 3.5 % NaOCl for 15 minutes and rinsed three times with autoclaved water. A 10 cm root sample per study plant was cut aseptically into 1 cm pieces in a laminar flow hood. The pieces were plated on modified ½ MMN medium (Marx 1969). The plates were incubated in room temperature in darkness and checked at regular intervals for emerging fungal colonies. When an outgrowing fungal colony was observed, it was immediately transferred to a new plate to obtain an isolated pure culture (i.e., isolate). The isolates were morphotyped based on macromorphological characteristics, such as growth rate, colour and surface structure.

Winter incubation experiment setup (Fig. 1)

One litre growth pots were rinsed with 70 % ethanol and filled halfway with autoclaved sand (8 L of sand was autoclaved for 4 h). To control the root sample separation from the substrate at the end of winter-incubation, roots were placed into perforated plastic bags (7 cm × 7 cm, SEFARNITEX 06-500/47, Ravatek Oy/Ltd), mesh size 0.5 mm. The bags were placed onto the sand in pots and further covered with a 1-cm layer of sand. The pots were placed on trays, 10 pots/tray and the trays were covered with tissue paper to reduce airborne contamination during the winter incubation. The trays were placed in dark conditions at 0-2 °C for five months. During the winter incubation, the pots were kept moist by adding 2 cm water to the trays every 4–6 weeks. Three weeks before the end of the winter incubation, the pots were moved into greenhouse conditions (20–21 °C day/17–18 °C night, 18 h light/6 h dark, lamps 400 W Philips SON-T PIA Green Power) to facilitate the recovery of fungi.

Identification of isolate morphotypes

Initially, classification based on macromorphological characteristics resulted in the identification of 23 isolate morphotypes. In addition, colonies characterized by slimy outgrowths were considered as yeast-like and counted separately. Further identification was carried out through ITS sequencing and phylogenetic analysis. DNA was successfully obtained for 14 morphotypes, generating a total of

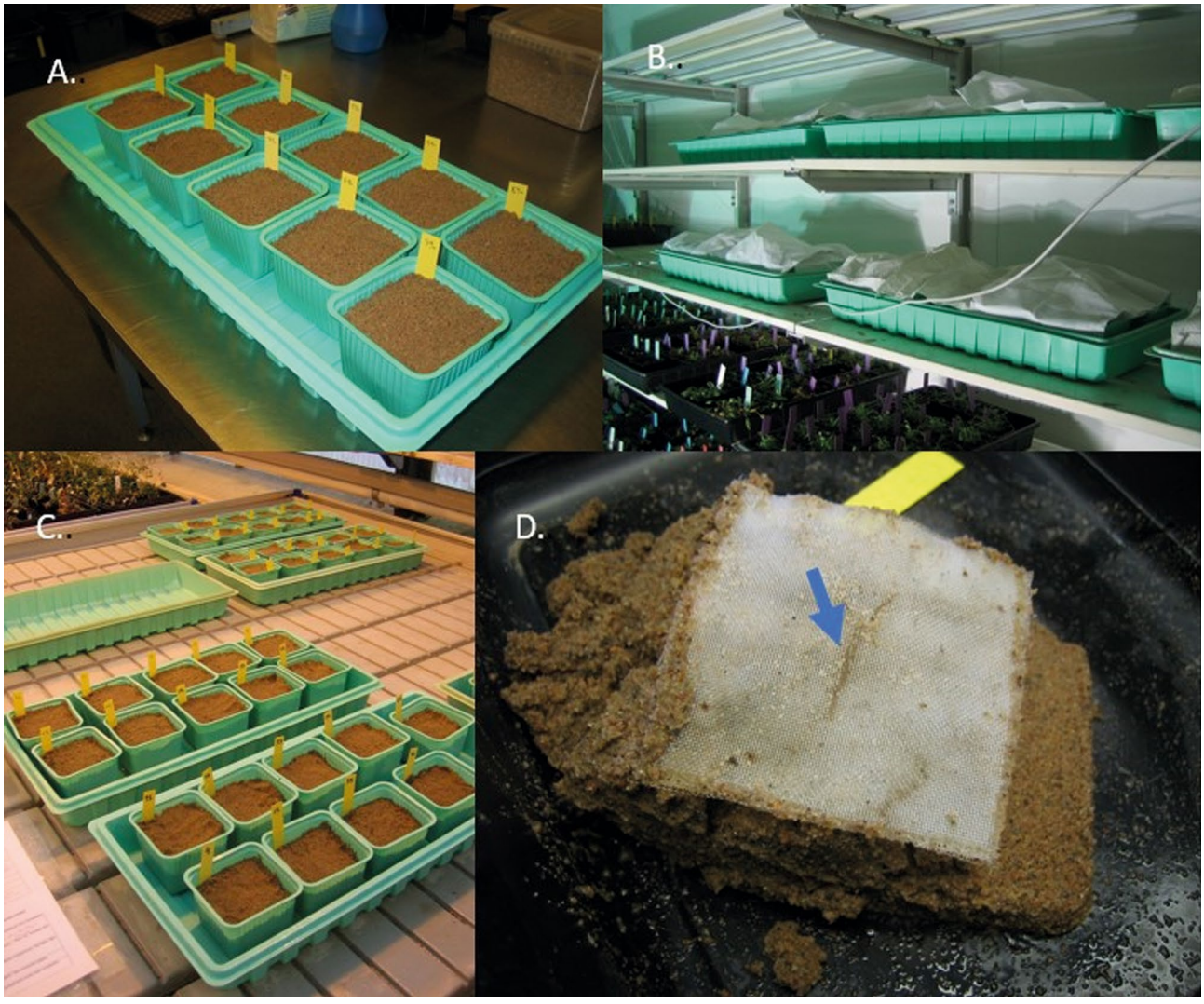

Fig. 1. **A.** Pots filled with sand ready for winter incubation. **B.** Winter incubation conditions in the cold room. **C.** At the end of winter incubation, the pots were kept on a greenhouse table for three weeks before harvest to simulate the start of the growing season. **D.** Example of a root sample (arrow) inside polyester bag at harvest.

16 DNA samples, including duplicates. DNA extraction from 0.5 to 1.0 g of fresh mycelia followed the protocol outlined by Pirttilä et al. (2001). The specific rDNA region, encompassing ITS1 and ITS2 regions along with the 5.8 S gene, was targeted for amplification using ITS1 (TCCGTAGGT GAACCTGCGG) and ITS4 (TCCTCCGCTTATTGA TATGC) primers, as described by White et al. (1990). The amplification process took place in a total reaction volume of 25 µl, comprising 2 mM of each dNTP, 2.5 mM MgCl2, 5 pM of each primer, 1 unit of Taq DNA polymerase (Dynazyme, Finnzymes, Espoo, Finland), and 100 ng of template DNA.

PCR amplifications were conducted in a thermal cycler (PTC 200, MJ Research, Watertown, MA, USA) with an initial denaturing step at 94 °C for 3 min, followed by 35 amplification cycles involving steps at 94 °C for 60 s, 50 °C for 60 s, and 72 °C for 120 s. A final extension step at 72 °C for 10 min concluded the amplification process. The resulting products were electrophoresed at 100 V for 1 h in 1× TAE buffer on a 1.4 % (w/v) agarose gel, stained with ethidium bromide (0.5 µg/ml), visualized under 300 nm UV light, and photographed. A 100-bp size marker (MBI Fermentas, Vilnius, Lithuania) served as a reference during the process.

Sequencing of fungal ITS region

The amplification products resulting from PCR reactions using unlabelled ITS primers (ITS1 and ITS4) were employed for the sequencing process. Sequencing reactions were carried out utilizing the Big Dye Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems (Foster City, CA, USA), following the manufacturer's instructions. The extension products were subsequently purified using the ethanol/EDTA precipitation protocol and subjected to analysis on the ABI 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) as per the manufacturer's recommendations. For each isolate, DNA sequences obtained from both the forward (ITS1) and reverse (ITS4) primers were individually inspected for quality. The two strands of DNA were then assembled using Sequencher 4.7 software (Gene Codes Corporation, Ann Arbor, MI, USA) to generate a consensus sequence for each isolate. These sequences were submitted to the National Center for Biotechnology and Information, and accession numbers were acquired.

Molecular phylogenetic analysis

All obtained sequences underwent a BLASTn search to compare them with ITS sequences available in GenBank. The closest matches found in GenBank were incorporated into a Clustal alignment, and the alignment process was carried out using ClustalX with default settings, as outlined by Thompson et al. (1997). Subsequently, a phylogenetic analysis was conducted using the maximum parsimony method through Molecular Evolutionary Genetics Analysis (MEGA11), as described by Tamura et al. (2021) and Stecher et al. (2020). To assess the confidence in specific clades resulting from the topology, bootstrap analysis with 1,000 replicates was performed. Additionally, tree scores, including the consistency index (CI), retention index (RI), and rescaled consistency index (RCI), were calculated for all trees. The MP tree was generated using the Subtree-Pruning-Regrafting (SPR) algorithm with a search level of 1, involving the initial acquisition of trees through the random addition of sequences in 10 replicates. The final dataset encompassed a total of 1339 positions.

Statistical analysis

The total number of fungal isolates, the number of isolate morphotypes and the number of yeast-like fungal growth were analysed with linear mixed effects model (lme-function) with plant species (levels: *Avenella*/*Lysimachia*) and season (levels: autumn/spring) as explanatory variables. The original field site and the individual plant specimen were used as random factors in the models with specimen nested within the site. The fit of the models was evaluated by using residual plots. We visualized the community structure of the endophytes by using NMDS plots associated with multivariate analyses PERMANOVA (adonis2-function) and PERMDISP (betadisper-function) analyses; to test the community differences (centroids) and homogeneity (dispersal), respectively. The analyses were carried out with the packages *emmeans, nlme and vegan* using R versions 4.1.0 (R Core Team 2021) and 4.2.2. (R Core Team 2022).

Results

Based on visual examination, roots of both plant species were covered with dark fungal hyphae after the winter incubation. *Lysimachia* roots were in a more degraded condition compared to their condition in the autumn whereas in *Avenella* there was no observable difference between autumn and spring samples based on dissection microscope observations.

We found that the total number of fungal isolates growing from *Avenella* roots was higher than the number of isolates growing from the roots of *Lysimachia* (Fig. 2a, Tab. 1). For *Avenella*, the number of isolates was higher after winter incubation compared to the number of isolates in the previous autumn whereas in *Lysimachia* there was no statistically significant difference (Fig. 2a). The number of isolate morphotypes was also higher in *Avenella* than in *Lysimachia* (Fig. 2b, Tab. 1). The number of isolate morphotypes was higher in the autumn than in spring for *Avenella*, whereas there was no difference in the number of morphotypes in *Lysimachia* (Fig. 2b, Tab. 1). We also found that mucoid, unicellular colonies, representing bacteria or yeasts (termed "yeast-like"), were more commonly isolated from *Avenella* than from *Lysimachia* (Fig. 3, Tab. 1). In *Avenella*, the number of mucoid isolates was higher in the spring than in the autumn, whereas there was no difference in *Lysimachia* (Tab. 1, Fig. 3).

Among the 16 sequences subjected to BLAST search, only two could be specifically identified at the species level. These were two isolates of *Phialocephala fortinii* (DF7 and TE14) and *Meliniomyces variabilis* (TE2). One isolate was assigned to the Helotiales order, whereas the rest of the sequences remained unidentified fungal sequences. Fungal

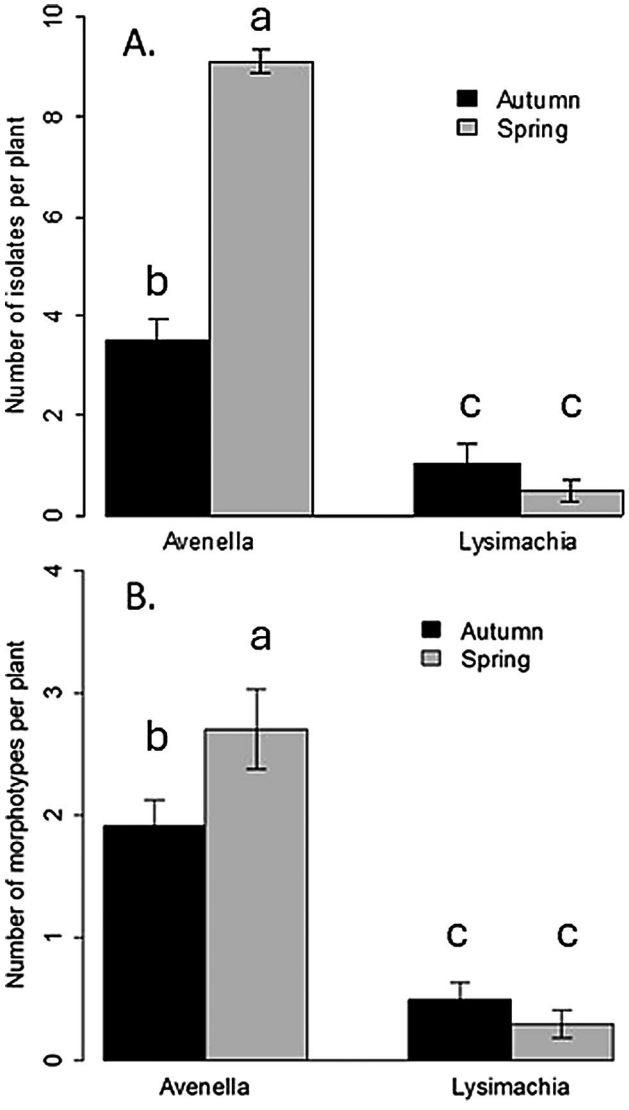

Fig. 2. Total number of fungal isolates (**A**) and of isolate morphotypes (**B**) (±SE) in *Avenella flexuosa* and *Lysimachia europaea* prior to and after winter incubation (autumn and spring). Letters denote the differences between bars (P<0.001, except for *Avenella* isolate morphotypes autumn vs. spring P=0.02).

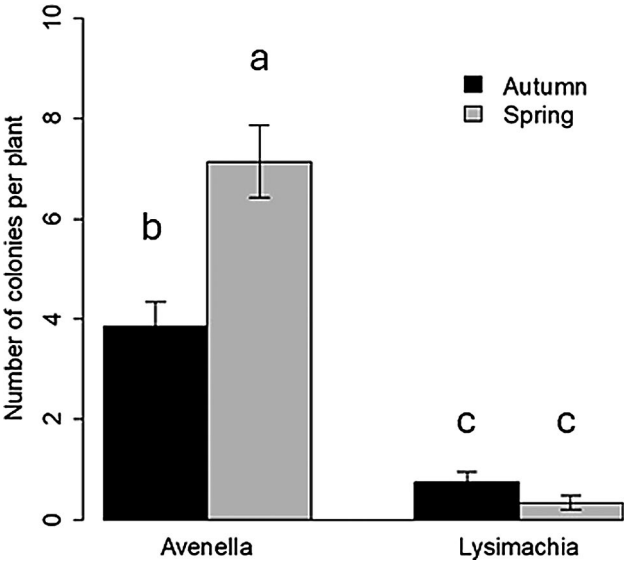

Fig. 3. Percentage of roots showing yeast-like colony outgrowth from *Avenella flexuosa* and *Lysimachia europaea* roots prior to and after winter incubation (autumn and spring). Letters denote the differences between bars (P<0.001).

community of *Lysimachia* was dominated by isolates identified as *Phialocephala fortinii* (49 % of isolates) whereas *Phialocephala* isolates consisted of only 4 % of all isolates in *Avenella*.

For the phylogenetic analysis, the Maximum Parsimony (MP) method was employed, resulting in the depiction of the most parsimonious tree with a length of 1044. All 16 sequences obtained in the present study were included in the phylogenetic analysis. The consistency index was calculated as 0.755513 (0.651163), the retention index stood at 0.654472 (0.654472), and the composite index was determined as 0.494462 (0.426168) for all sites and parsimony-informative sites.

The NMDS and community multivariate analyses indicated differences in the isolate morphotype communities (PERMANOVA: host species F=3.63,

Tab. 1. Impact of plant species (*Avenella flexuosa*/*Lysimachia europaea*) and season prior to and after winter incubation (autumn/spring) and their interaction (*) on the total number of root fungal isolates, the number of isolate morphotypes and the number of yeast-like colonies based on linear mixed effects model. F = test statistic, df = degrees of freedom, P = p-value.

	Number of isolates			Number of morphotypes			Number of colonies		
	F	df	P	F	df	P	F	df	P
Species	247.9	1,45	<0.001	65.6	1,45	<0.001	116.1	1,42	<0.001
Season	66.8	1,46	<0.001	2.5	1,46	0.123	11.5	1,43	<0.01
Species*season	98.2	1,46	<0.001	7.27	1,46	<0.01	22.8	1,43	<0.001

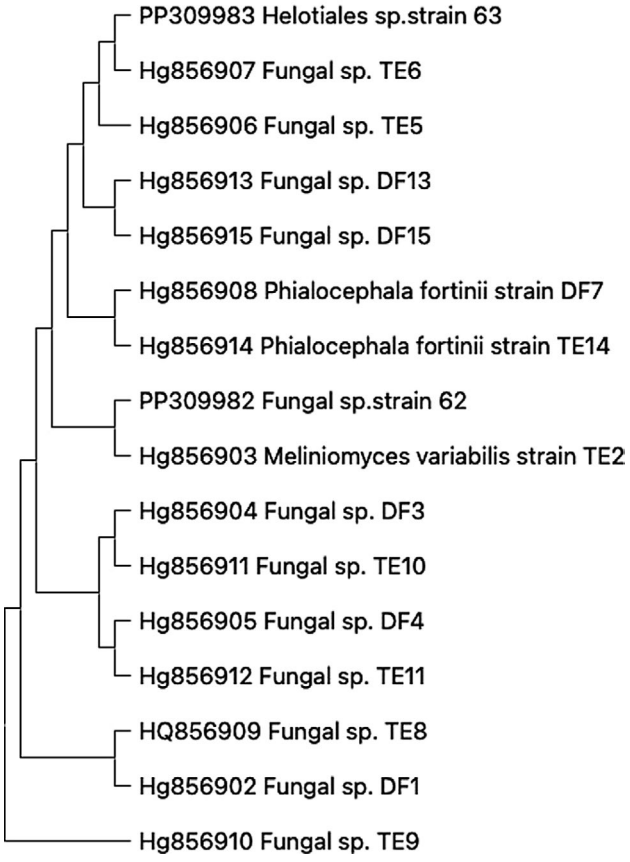

Fig. 4. Phylogenetic analysis of root endophytic fungi of *Avenella flexuosa* and *Lysimachia europaea* by analyzing their ITS and 5.8 S rDNA sequences. The phylogenetic tree was constructed using 16 endophytic fungal sequences obtained from the study. Accession numbers and names accompany the sequences sourced from GenBank.

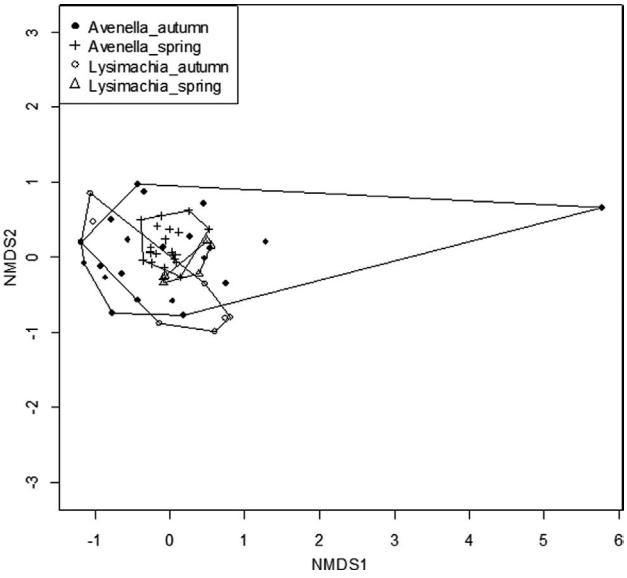

Fig. **5**. Root fungal endophyte community composition (NMDS ordination) based on isolate outgrowth of *Avenella flexuosa* (Avenella) and *Lysimachia europeaea* (Lysimachia) prior to and after simulated winter incubation of roots (autumn, spring).

P<0.001, season F=9.78, P<0.001 and host species*season F=2.99, P<0.01, df 1,55 and PERMDISP: F=3.83, P=0.03, df 3,55). Pairwise comparisons of PERMDISP suggest that isolate morphotype community homogeneity differed between *Avenella* autumn and spring (P<0.001), *Avenella* and *Lysimachia* autumn (P<0.001), *Avenella* autumn and *Lysimachia* spring (P<0.001) and *Avenella* autumn and *Lysimachia* spring (P=0.01). There was no difference in isolate morphotype community homogeneity between *Avenella* and *Lysimachia* spring as well as between *Lysimachia* autumn and spring (NS) (Fig. 5).

Discussion

We found higher total numbers of isolates and higher numbers of isolate morphotypes in *Avenella* roots than in *Lysimachia* roots both prior to and after a simulated winter (i.e., autumn and spring, respectively). In *Avenella,* more fungal outgrowth and higher numbers of isolate morphotypes were observed in the spring than in the autumn, whereas in *Lysimachia* the culturable fungal community did not differ between autumn and spring.

Although the community structure of the fungal endophytes became more uniform through the winter-period, the endophyte communities still maintained host-species specificity. The identity of the host may be an important determinant of fungal endophyte communities (e.g., Botnen et al. 2020, Kivlin et al. 2022). Some previous studies have indicated that different plant species carry contrasting root endophytic fungal communities, even when living in a close proximity next to each other (Wearn et al. 2012, Tejesvi et al. 2013, Brigham et al. 2023). In this study, the fungal community of *Avenella* roots, including the yeast-like outgrowth, was more diverse than that of *Lysimachia*, both prior to and after the simulated winter treatment (see also Tejesvi et al. 2013). This is in agreement with previous observations stating that seasonal shifts in root fungal communities, including mycorrhizal fungi, are host-specific (Li et al. 2005, Ruotsalainen et al. 2002, Wearn et al. 2012).

Our results suggest that the root endophytic fungi of *Avenella* and *Lysimachia* can overwinter in decaying roots until the next growing season, as we

detected similar fungal isolate morphotypes both in the autumn and spring (although this was not always the case at the individual root sample level). Survival of the root-endophytic fungi through the winter or another unfavourable season can take place via many mechanisms, although the information to date is largely circumstantial. In the case of DSE fungi, it is known that they form intraradical microsclerotia, which, in addition to melanized hyphae of DSE, have been assumed to play a role for survival and dispersal of these fungi (Currah et al. 1993, also reviewed by Jumpponen & Trappe 1998). DSE-type microsclerotia have also been found more commonly in dormant and inactive roots than in roots of physiologically active plants (Barrow & Aaltonen 2001). Microsclerotia are reported to include glycogen, proteins, and polyphosphate (Yu et al. 2001) suggesting that microsclerotia have a storage function. Microsclerotia potentially contribute to the survival of endophytic fungi over winter or other unfavourable seasons.

Our results also suggest that root endophytic fungi of *Avenella* and *Lysimachia* may represent hitherto unknown species. There were only two fungi identified at the species level, *Phialocephala fortinii* and *Hyaloscypha (Meliniomyces) variabilis (*Fehrer et al. 2018*)* of which *P. fortinii* was found in both plant species. *Meliniomyces variabilis* is a relatively recently described fungus involved in ericoid mycorrhizal symbiosis and potentially also in other mycorrhizal types (Hambleton & Sigler 2005, Ohtaka & Narisawa 2008). Identification of root-colonizing fungal communities of *Avenella* and *Lysimachia*, as well as other root-associated fungi in the boreal forest field layer needs work including developing culturing and sequencing techniques, as well as preservation and identification of fungal sporocarps as reference material, when possible (Tanney & Seifert 2020).

Lysimachia showed a strikingly narrow root endophyte diversity compared to the diversity in *Avenella*. In addition, *Phialocephala fortinii*, a fungus associated with the DSE-group (Jumpponen & Trappe 1998) and belonging to the PAC-complex (Stroheker et al. 2024) dominated in *Lysimachia*. *Lysimachia* and *Avenella* have different life forms: *Lysimachia europaea* is a pseudoannual herb, which suggests that basically all the belowground structures of a single ramet, except daughter tubers, can potentially decay during the winter period (Taylor et al. 2002). *Avenella flexuosa*, in contrast, is a perennial, tussock-forming grass. These different life form strategies of the host could affect the overwintering options of the root endophytic fungi, but this aspect cannot be evaluated with the present data.

We conclude that culture-based approach allowed us to observe fungal endophyte status of detached *Avenella flexuosa* and *Lysimachia europaea* roots prior to and after a simulated winter incubation. Our results suggest that root fungal endophyte communities undergo changes during simulated winter but stay largely host-species-specific during and after the winter incubation. *Avenella* roots possessed a taxon-rich and more diverse fungal community whereas the fungal community of *Lysimachia* remained narrow and more unchanged from autumn to spring. We conclude that the fungal community of the decaying root fragments of these plant species may remain viable until the beginning of the next growing season and decaying roots and their fragments have potential to act as an inoculum source of root endophytic fungi. We further suggest that the host-specific differences in root fungal communities have potential to contribute to the fungal community dynamics in soil and surrounding vegetation.

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