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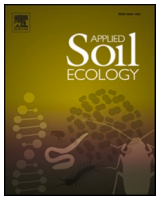

Fungal and arbuscular mycorrhizal communities unique to old grasslands in a Swedish agricultural landscape

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ABSTRACT

Fungal and arbuscular mycorrhizal communities in semi-natural grassland soils depend on management regime, but we lack knowledge about these communities in a north European agricultural landscape context. Several species inhabiting semi-natural grasslands are of a high conservational interest, but their presence in nearby short-term grasslands such as leys is unknown. We investigated fungi and arbuscular mycorrhizal fungi (AMF) using DNA metabarcoding and quantitative PCR in soils and roots in nine semi-natural grasslands and adjacent leys and assessed unique and overlapping community members and their guilds. We observed a generally higher abundance and alpha diversity of total fungi and AMF in grasslands than in leys, but only in the uppermost soil layer. At the landscape level, leys also had more similar fungal and AMF communities than grasslands. Both fungal and AMF community composition differed between grasslands and leys, with higher relative abundance of root-associated ascomycetes, saprotrophic basidiomycetes and AMF *Glomeraceae* in grasslands, and more pathogens and dung saprotrophs in leys. The fraction of species shared between soils and roots was higher in grasslands than in leys. We identified distinct fungal and AMF communities associated with semi-natural grasslands that could be of interest for conservation purposes. Assessing how these communities respond to management will be important for proposing conservational measures. The higher abundances of saprotrophic basidiomycetes and root-associated ascomycetes in grasslands than in leys, together with a greater overlap of species between soils and roots, suggests that processes in soils may be more interconnected with roots via fungi in semi-natural grasslands.

1. Introduction

Soil fungi contribute to soil biodiversity and mediate several ecosystem processes such as nutrient cycling and plant performance across contrasting ecosystems (Tedersoo et al., 2014; Van Der Heijden et al., 2008). Yet, there is a knowledge gap concerning fungal diversity and functional roles in semi-natural grasslands (hereafter referred to as grasslands) in landscapes dominated by agriculture in Northern Europe, although they are recognized as important for biodiversity conservation and listed in the EU Habitats Directive (Halada et al., 2011). Grasslands often harbour a high diversity of plants, birds, earthworms and pollinators (Aguilera Nuñez et al., 2024; Lindborg et al., 2008; Dengler et al., 2014; Söderström et al., 2001; Torppa et al., 2024; Wilson et al., 2012), and can be important for the provisioning of ecosystem services such as water regulation, pollination, feed production, cultural heritage (Bengtsson et al., 2019) as well as soil carbon sequestration (Bai and Cotrufo, 2022). Grasslands are sustained by herbivore grazing or mowing (Glimskär et al., 2023; Queiroz et al., 2014) and optimization of management could further enhance biodiversity and the ecosystem services they provide (Bai and Cotrufo, 2022; Norderhaug et al., 2023). However, the lack of knowledge of the belowground soil biota inhabiting grasslands is hampering our ability to elaborate efficient management practices aiming at biodiversity conservation.

Fungal and arbuscular mycorrhizal fungi (AMF) are essential

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organisms for soil organic matter recycling and plant nutrient uptake. Soil fungi are functionally diverse: they are main drivers of soil organic matter transformations but can also act as pathogens and mutualists of plants (Baldrian, 2017). Although AMF have limited capacities to decompose organic matter, they are important for plant nutrient uptake — particularly phosphorus — and often improve plant performance (Van Der Heijden et al., 2008). Grassland soils have been found to harbour different communities and often disproportionately higher diversity of fungi and AMF compared to forests (Labouyrie et al., 2023; Öpik et al., 2006) and arable land (for AMF, Öpik et al., 2006). Grasslands have also gained focus as important habitats for certain saprotrophic basidiomycetes, particularly waxcaps (*Hygrocybe* spp.), several of which are included on the IUCN Red List (Griffith and Roderick, 2008; Dahlberg and Mueller, 2011). A recent global fungal red list assessment shows that grasslands are the second habitat after forests in terms of number of threatened fungal species (Mueller et al., 2022). This is of concern since, globally, and particularly in Northern Europe, grassland cover has declined significantly (Eriksson et al., 2002; Pe'er et al., 2014), mostly due to land use changes (Griffith et al., 2013; Eriksson et al., 2002; Queiroz et al., 2014). However, our knowledge of the distribution of waxcaps and other threatened fungal species is mainly based on fruiting body occurrences, and it remains to be investigated whether these endangered species occur in mycelial state in adjacent croplands. Understanding how fungal and AMF communities are distributed in grasslands versus more disturbed croplands can inform conservation strategies and reveal functional roles. For example, certain fungi and AMF species may be particularly important for improved nutrient uptake in improved grasslands compared to the AMF that are sustained in crop rotations (Vogelsang et al., 2006; Edlinger et al., 2022). Effects of management on above-ground biodiversity, e.g. plants and pollinators, in grasslands has been frequently assessed (e.g. see (Berg et al., 2019)) but there is limited knowledge on fungi. Different management, such as intensive management in arable land (i.e. fertilization, and tillage), has been shown to change composition and reduce richness of fungal and AMF communities (Banerjee et al., 2019, 2024; Habekost et al., 2008; Oehl et al., 2004; Peltoniemi et al., 2021; Verbruggen et al., 2016). Particularly nitrogen and phosphorus addition (Ceulemans et al., 2019) and pesticides (Edlinger et al., 2022) has been shown to impact AMF. Management can also indirectly affect soil fungi by altering plant communities, especially fungi that establish symbiotic relationships with plant roots, such as AMF (Van Der Heijden et al., 2008; Edlinger et al., 2022). Although land use intensification of grasslands can decrease diversity and homogenize communities, including fungi (Gossner et al., 2016), extensive long-term management of grasslands based on low chemical inputs and minimal soil disturbance, can also enhance diversity and spatial heterogeneity of plant, bird and pollinator communities (Dengler et al., 2014; Söderström et al., 2001; Glimskär et al., 2023), but this still needs to be assessed for fungi. Grasslands vary in nutrient and moisture conditions, but also in mowing and grazing type and intensity (Söderström et al., 2001; Löfgren et al., 2020; Milberg and Tälle, 2023), which can potentially maintain diverse ecological niches of fungi across habitats. Differences in management could also promote habitat heterogeneity across the vertical soil profile since plant root traits, density and distribution respond to grazing type and intensity (Bonin et al., 2013). This can also influence how fungi and fungal plant symbionts are distributed in the soil vertical profile and interact with plant roots and the rhizosphere soil.

An important step to understand whether grasslands may function as belowground biodiversity hotspots and to adequately manage fungal species with conservation needs is to profile the fungal communities that are exclusively inhabiting these grasslands, versus communities in arable fields. In Sweden, grassland cover has decreased by 90 % (Eriksson et al., 2002) during the last century and currently represents 200,000 ha (Aguilera Nuñez et al., 2024) while 36 % of agricultural lands are leys, i. e. short-term (2–3 years) grasslands in crop rotations, representing ca 1,068,200 ha (Jordbruksverket, 2024). Although more intensively managed than grasslands, leys support a less disturbed crop rotation with year-round plant cover and a mix of species, which altogether could be a diversity gateway into the more managed systems, such as ploughed and fertilized crops. We investigated abundance, diversity and community composition of total fungi and AMF in soils and roots of nine grasslands and adjacent leys and assessed proportions of unique shared community members and their guilds. Our primary objectives were to i) test the hypothesis that grasslands harbour a higher abundance and alpha diversity of fungi and AMF than young leys. Additional aims were to ii) characterize differences in taxonomical and functional community composition, to compare the degree of community turnover of fungi and AMF across leys and grasslands at a landscape scale, and to iii) define fungal and AMF communities in roots that are unique to grasslands versus leys.

2. Material and methods

2.1. Study area and sampling sites

We used a subset of the grasslands from Torppa et al. (2024) and adjacent leys, giving a total of nine grassland-ley pairs, all situated in Uppland, Central Sweden, covering an area around 1400 km^2 (Fig. S1). The grasslands were selected based on previous vegetation surveys (Pärt and Söderström, 1999; Söderström et al., 2001) and the plots were based on historical maps and aerial photographs to inspect that no crop cultivation activities had occurred for at least the last 150 years. Within each grassland, we selected areas with similar vegetation type, indicating mesic and medium-fertile soils without influence of mineral fertilization. The grasslands were grazed by cattle, unfertilised and not mown, according to interviews with the farmers. Paired leys within the same farm were selected as close as possible, within 500 m of each grassland. The leys were part of rotational cropping systems, and they had last been ploughed 2–3 years previously, followed by sowing of ley plant mixtures, mainly *Trifolium* spp., *Poa* spp., *Lolium perenne*, *Dactylis glomerata*, *Festuca pratensis*, *Phleum* spp., and *Medicago sativa*. The study region consists mainly of low hills covered by bedrock outcrop or moraine, and glacial clays or organic soils in the lower parts. Climate in the study area is humid continental, with a mean annual temperature of 6.8 °C and a mean annual precipitation of 541 mm over the past 30 years (Swedish University of Agricultural Sciences, Ultuna Weather Station, 1991–2020). The grasslands were generally located on small hills with coarser soil textures, whereas the leys were typically located on finer sediments at slightly lower positions.

2.2. Sampling and soil characteristics

In each grassland and ley, we established 3 circular plots (3 m radius), 10 m apart and collected 5 soil cores in each plot (>1 m apart; 15 soil cores in total in each site) using a cylindrical steel corer of 3 cm in diameter. Samples were taken down to 20 cm. The cores were divided into two depth layers, 0–10 cm and 10–20 cm, and the 15 samples pooled per layer and site. Samples were brought to the laboratory, weighted the same day, and kept at −20 °C until further processed. The entire sample volumes were first milled while kept frozen and then immediately stored at −20 °C until further processed. A sub-sample (around 150 g) of the homogenized soil was weighted and freeze-dried for 72 h, after which soil moisture content was measured. The freeze-dried soil was then sieved (2 mm), and large debris and stones were removed and weighted. The resulting sample was then ground to a fine powder using mortar and pestle and used for DNA-based analyses. Roots were obtained from another 150 g of frozen and milled soil subsample (only 0–10 cm depth layer), which was carefully rinsed with water on a 0.5 mm mesh. The resulting root samples were inspected under a stereo microscope, and remaining debris and other non-root materials were removed. The mixed root samples were freeze-dried for 72 h. A scheme summarizing sample handling and processing is shown

in Fig. S2.

To obtain information on soil type and fertility, another subsample of the homogenized fresh sample was thawed and analysed for soil texture (clay, silt and sand) and pH at Agrilab Uppsala, Sweden, following the Swedish standards SS-ISO 11 277 and SS-ISO 10 390, respectively. Freeze-dried and milled subsamples were analysed for organic matter content by loss-on-ignition at 550 °C for 6 h. Total carbon (C) and nitrogen (N) content and $\delta^{15}N$ was determined using an isotope ratio mass spectrometer (DeltaV, Thermo Fisher Scientific, Bremen, Germany) coupled to an Elemental analyser (Flash EA 2000, Thermo Fisher). Total C and N stocks were calculated by multiplying contents (%) with soil dry mass per unit area (g m^{-2}) for each depth layer.

2.3. DNA extraction and quantification of microbial abundance

DNA was extracted from soil and roots. For soil, DNA was extracted from 4 g of freeze-dried and milled soil using the DNeasy ® PowerMax ® Soil Kit following the manufacturers' protocol. DNA concentration was measured on a Qubit Fluorometer (Life Technologies). DNA was also extracted from 50 mg of freeze-dried and milled roots using NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany). We used larger DNA extraction volumes for soils because of the patchy distribution of AMF fungi in soils.

Total fungal abundances were quantified using qPCR with SYBR Green on a BioRad CFX Connect Real-Time system, by targeting the internal transcribed spacer region 2 (ITS2) using the primers fITS7 (Ihrmark et al., 2012), ITS4 (White et al., 1990) and ITS4arch which covers Archaeorhizomycetes (Sterkenburg et al., 2018). The reaction mix of 20 µl included BSA (0.1 %) and iQ-SYBR Green (1×), 0.5 µM (gITS7), 0.3 µM (ITS4), and 0.15 µM (ITS4A), and 4 ng of soil DNA. The annealing temperature was set to 56 °C. Prior to quantification, all samples were tested for inhibition by amplifying 1×10^5 copies of pGEM plasmid (Promega, WI, USA) spiked into qPCR reactions with either soil DNA or non-template controls, together with plasmid-specific primers (M13F and M13R). No inhibition was detected in any of the samples for the amount of DNA used. A standard was prepared consisting of serial dilutions of known amounts of a linearized plasmid containing the target fragment. Samples were run in duplicate in two different runs, and results were repeated if coefficient of variation between duplicates was >30 %.

2.4. Fungal and arbuscular mycorrhizal communities

Fungal communities in soils and in roots were PCR amplified in duplicates using the same primers as in the qPCRs, but with the addition of 8-base sample-specific identification tags (Clemmensen et al., 2023). AMF communities in soils were amplified using the primers using the primers NS31 (Simon et al., 1992) and AML2 (Lee et al., 2008). The PCR reactions of 50 µl contained 12.5 ng (fungi) or 75 ng (AMF) of template DNA. Fungal amplifications were done with minimized numbers of PCR cycles to avoid length-based biases (between 24 and 28 cycles, Castaño et al., 2020), while AMF amplifications were run using the same number of cycles because fragments do not vary in length. For both AMF and fungi, the reaction mix contained 200 µM nucleotides, 2.75 mM $MgCl_2$, and primer concentrations as in the respective qPCR reactions. Resulting amplicons were purified using AMPure (Beckman Coulter, Beverly, MA, USA) and concentrations were measured fluorometrically (Qubit high sensitivity kit, Invitrogen, Carlsbad, CA, USA). Equal DNA amounts from each sample were pooled, further cleaned using the Cycle Pure kit (EZNA, Omega Bio-Tek, Nocross, GA, USA), and then amplicon sizes were checked using Bioanalyzer (Santa Clara, CA, USA). The final two pools were sequenced on a Pacific biosciences Sequel I system using one SMRT cell per pool.

The SCATA pipeline (https://scata.mykopat.slu.se/, Last accessed 26th Sept 2022) was used to perform sequence quality filtering and clustering for both fungi and AMF. For quality filtering, reads that contained bases with a quality score lower than 10, had an average read quality of <20 or were shorter than 200 bases were discarded. Reads missing sample-identification tags (100 % match required) or did not have at least a 90 % match with primers sequences were discarded. Before clustering, homopolymers were reduced to three bases. Both for fungi and AMF, species-level clustering of high-quality sequences was based on single-linkage clustering with a minimum similarity of 98.5 % to the closest neighbour required to enter a cluster. After rank abundance comparisons between communities obtained using single-linkage clustering and amplicon sequence variants obtained from DADA2, the clustering threshold for AMF was set at 99.5 %. This threshold allowed us to separate more virtual taxa (Öpik et al., 2010), thus both for fungi and AMF operational taxonomic units (OTUs) were obtained. All fungal species were annotated using the species hypothesis matching option in the UNITE database (Abarenkov et al., 2024). The 550 most abundant species were also manually checked using massBLASTer in the UNITE PlutoF module. Fungal species were classified into guilds using FungalTraits (Põlme et al., 2020) and manually curated based on the massBLASTer output. For AMF, sequences were assigned to virtual taxa using the MaarjAM database (Öpik et al., 2010) but non-AMF taxa were discarded and OTUs were taxonomically identified using Eukaryome database (Tedersoo et al., 2024). Thorough the manuscript, the term species is used for both fungal species hypothesis and AMF virtual taxa. Non-fungal or non-AMF species were omitted from the root communities (35 % non-AMF reads and 18 % non-fungal reads) and for the soil communities (31 % non-AMF reads and 17 % non-fungal reads).

2.5. Statistical analyses

All statistical analyses were done using the software R (version 4.1.3, R core Team, 2022). Two-way ANOVAs were used to test main effects and interactive effects of soil depth (0–10 and 10–20 cm) and system type (leys and grasslands) on soil parameters ($\delta^{15}N$, C and N stocks, C:N ratio, pH, and texture) and microbial abundances (fungi, AMF). If interactions were significant, tests were done separately for each soil depth layer. Data was log or square-root transformed when assumptions of normality and homoscedasticity were not met, and Kruskal-Wallis test was performed when transformation did not meet assumptions.

For diversity estimates, we calculated Hill numbers as implemented in the iNEXT function in R (Hsieh et al., 2016). Shortly, Hill numbers are unified diversity metrics and differentiated by the order of q, which defines the weight given to rare vs. dominant species. For $q = 0$, Hill numbers equal species richness (counting all species equally), Hill numbers with $q = 1$ reflect the exponential of the Shannon entropy (emphasizing all species according to their proportion in the community), and with $q = 2$ Hill numbers are the inverse Simpson index (dominant species are emphasized). We obtained the Hill numbers corresponding to a sequencing depth of 400 reads per sample. Hill numbers were extrapolated using the same function for two samples that did not have enough reads. Effects of grassland vs ley system (S) and the interaction between system and soil layer ($S \times L$) on Hill numbers were tested by ANOVA. A function to estimate cumulative species richness across randomized samples was generated and used to plot cumulative richness across increasing numbers of sites for the two systems.

For fungal and AMF beta-diversity analyses, community data was Hellinger transformed. Bray-Curtis distances were calculated and Permutational multivariate analyses of variance (PERMANOVA) was conducted using the *adonis2* function to assess the effects of system type and soil depth layer, including their interactions. Beta dispersion tests were carried out to evaluate homogeneity of multivariate dispersion using the betadisper function. We used non-metric multidimensional scaling (NMDS) to visualize the ecological distances between samples. Differences in relative abundances of guilds (fungi) or families (AMF) between system types and layers were tested with ANOVA and response variables were transformed when normality and homoscedasticity assumptions were violated. Pair-wise DESeq2 analyses (version 1.26.0;

Love et al., 2014) were performed to detect differential taxa abundances between the two systems ($p < 0.05$), with soil depth layer defined as principal fixed factor in the analysis to account for the spatial replicates. Venn diagrams were obtained by identifying unique and shared species within and between systems and compartments (roots and soil). This was done by aligning species by their species hypothesis code (fungi) or virtual taxon code (AMF) across systems and compartments.

2.6. Accession numbers

Sequence data are archived at NCBI's Sequence Read Archive under accession number PRJNA1263165.

3. Results

3.1. Soil characteristics

Grasslands had higher C:N ratios ($P = 0.038$), N ($P = 0.005$) and C stocks (P = 0.005) in the upper 0–10 cm soil layer as compared to leys (Table 1). Root biomass was almost 5 times higher in grasslands than in leys ($P < 0.001$) in the upper soil layer. By contrast, the $\delta^{15}N$ signature and pH were higher in leys ($P = 0.024$ and $P = 0.04$, respectively). Soil texture also varied between systems, with overall higher clay content in leys and higher sand content in grasslands (P < 0.001; Table 1). For the 10–20 cm depth layer differences were smaller and only the C:N ratio was higher in grasslands ($P = 0.048$) and clay content was higher in leys ($P = 0.013$).

3.2. Abundances of fungi and AMF and fungal diversity and composition

There was a significant system type × layer interaction for fungal ($P < 0.005$) abundance. In the upper soil layer, abundance of fungi ($P = 0.009$) were higher in grasslands than in leys, while in the lower layer no significant differences were found between system types (Fig. 1).

Cumulative fungal and AMF richness with increasing numbers of sampled sites increased more across grasslands than across leys, and in a similar manner in both layers, indicating that communities were more homogenous in leys at the landscape level (Fig. 2). Richness was also generally higher in the uppermost layers, although in the ley systems AMF richness was similar in the two soil layers (Fig. 2c).

The alpha diversity of fungi was higher in grasslands than in leys in the upper soil layer and in roots (Fig. S3, $P < 0.05$ for all diversity metrics). The alpha diversity of AMF was also higher in grasslands in both soil layers (Table S1, P < 0.05), but not in roots where it was similar. Overall, significantly higher diversity was also observed in the uppermost soil layer compared to the deeper layer (Fig. S3, Table S1, P < 0.05).

Both total fungal and AMF communities differed in composition in grasslands compared to leys ($P < 0.001$ and $R^2 > 15$ % for all), while there were no differences between soil layers ($P > 0.05$, Fig. 3). Multivariate dispersion was higher for fungal communities in grasslands than in leys in both layers ($P < 0.01$ for all), but it did not vary for AMF. Overall, in grassland soils, there were higher relative abundances of root-associated ascomycetes and saprotrophic basidiomycetes, while plant pathogens, yeast basidiomycetes, dung saprotrophs and mycoparasites were more abundant in leys (Fig. 3b). For roots, only root-associated fungi were more abundant in grasslands, while ascomycete saprotrophs were more abundant in leys (Fig. 3b). Arbuscular mycorrhizal *Glomeraceae* species were more abundant in grassland soils, while *Diversisporaceae* and *Paraglomeraceae* and *Polonosporaceae* were more abundant in leys. For roots, only *Archaeosporaceae* were more abundant in leys (Fig. 3b).

Table 1
Soil characteristics in grasslands and leys for two different soil layers (average ± SE, $n = 9$). Letters indicate significant differences between systems ($P < 0.05$), and contrasts are done only between system types for each horizon separately. ND = not determined.

Layer	0–10		10–20	
Type	Grassland	Ley	Grassland	Ley
C (%)	10.1 ± 1.6^a	4.8 ± 1.1^b	6.3 ± 1.5^a	0.3 ± 0.1^b
N (%)	0.6 ± 0.1^a	0.3 ± 0.1^b	0.4 ± 0.1^a	0.3 ± 0.1^a
C stocks (kg m^{-2})	6.1 ± 1.0^a	3.6 ± 1.7^b	3.1 ± 0.8^a	2.7 ± 1.7^a
N stocks (g m^{-2})	494.8 ± 80^a	309.2 ± 138^b	270.5 ± 73^a	245.2 ± 140^a
C:N ratio	12.3 ± 0.7^a	11.6 ± 0.7^b	11.6 ± 1.0^a	10.8 ± 0.6^b
$\delta^{15}N$ (‰)	5.2 ± 1.4^b	6.7 ± 0.5^a	6.9 ± 1.3^a	7.2 ± 0.8^a
pH	5.8 ± 0.4^b	6.3 ± 0.6^a	6.0 ± 0.5^a	6.3 ± 0.5^a
Clay (%)	13.9 ± 5.6^b	33.2 ± 8.8^a	20.2 ± 10.1^b	33.4 ± 10.1^a
Silt (%)	38.7 ± 7.5^a	40.6 ± 8.0^a	37.4 ± 6.5^a	39.2 ± 8.0^a
Sand (%)	34.3 ± 10.5^a	21.9 ± 10.9^b	35.2 ± 11.9^a	23.6 ± 11.5^a
Roots (g kg^{-1})	2.18 ± 0.57^a	0.46 ± 0.26^b	ND	ND

3.3. Fungal and AMF species associated to each system and compartment

Several fungal species were significantly associated with either grasslands or leys. For soils, all the root-associated fungi and basidiomycete saprotrophs that showed significant associations were associated with grasslands. These species mainly belonged to Archaeorhizomycetes, but also dark septate endophytes such as *Cadophora* sp. and *Leohumicola verrucosa*, the latter also found associated to grassland roots. The basidiomycete saprotrophs, *Entoloma sericeum*, *Hygrocybe* spp., *Gliophorus psittacina* and *Cuphophyllus virgineus*, were more abundant in grasslands. However, a different set of saprotrophic basidiomycetes were significantly associated to grassland roots, particularly *Mycena* species. Moulds were also exclusively found in grassland soils, mainly *Penicillium antarcticum* and *Mortierella globullifera*. For AMF, most of the species significantly associated to grassland soils were *Dominikia* and *Acaulospora* species, while *Polonospora* and *Funneliformis* spp. were associated to ley soils (Fig. 4). Interestingly, AMF species associated to grassland soils were rare but more numerous, while those associated with ley soils had a higher overall abundance. For roots, only seven AMF species were significantly associated to grasslands.

When analysing unique and shared species across systems (leys and grasslands) and compartments (roots and soils), we found a larger number of fungal taxa unique to grasslands than to leys, both for soil and root communities (Fig. 5a). The percentage of fungal species found only in their respective system type but shared between soil and roots were also higher for grasslands than for leys (6.6 % vs 1.3 %, respectively). The species detected in both grassland soils and roots were mainly saprotrophic basidiomycetes and ascomycetes, and the most common genus was *Mycena*, represented by five species. Similar patterns were found for AMF, but with proportionally larger differences between systems and compartments (Fig. 5b). Of total fungi and AMF, 5.4 % and 22 %, respectively, were present in both grasslands and leys and in both compartments (Fig. 5).

4. Discussion

4.1. Main patterns in fungal and AMF communities between system types

Fungal and AMF diversity, composition, and abundance in both root- and soil-associated communities differed between adjacent leys and grasslands across this northern agricultural landscape. Diversity was overall higher in grasslands, which paralleled higher fungal abundances, particularly in the upper soil layer (0–10 cm). At the landscape scale, we also observed a disproportional accumulation of new species with more sampling effort in grasslands compared to leys suggesting overall more heterogeneous communities among grasslands than among leys. More unique fungal and AMF species were found in grasslands compared to leys, and a higher proportion of the species found in grasslands were present in both roots and soils. As expected, differences in soil

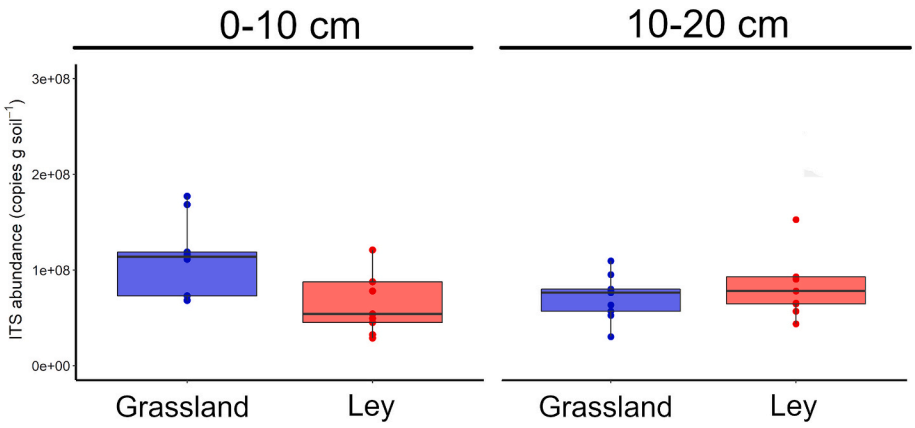

Fig. 1. Differences in fungal abundances between grasslands and leys in two soil layers.

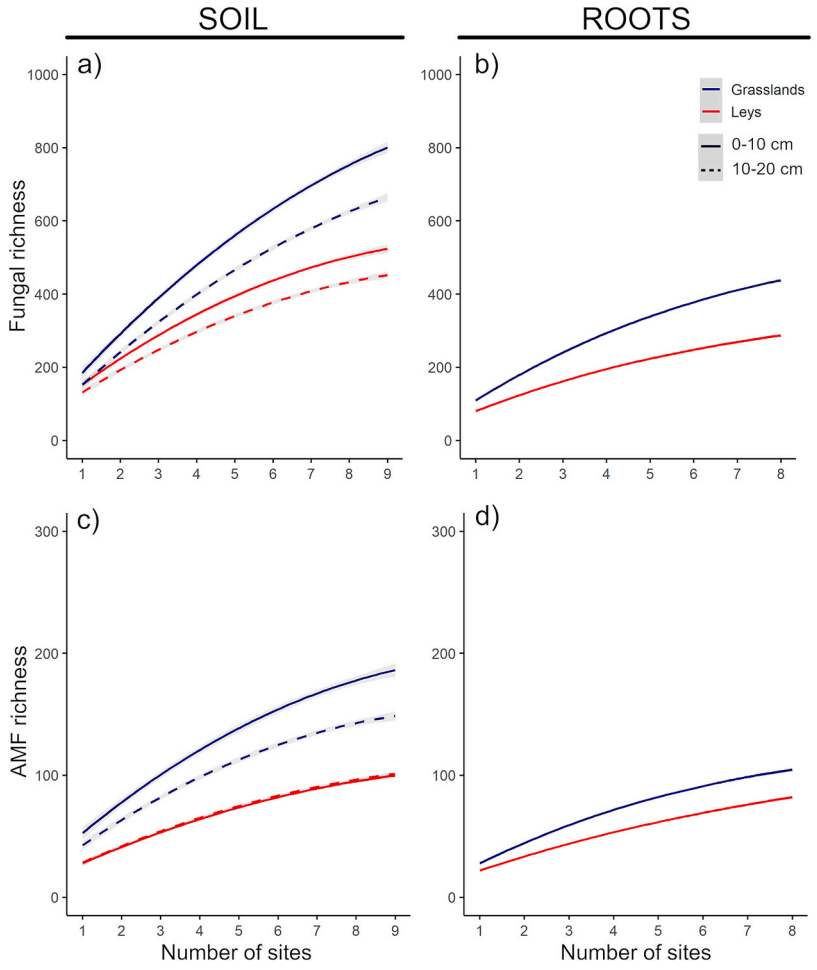

Fig. 2. Cumulative richness across all sampled sites for grasslands (blue) and leys (red) for the soil fungal community in a) soils and b) roots, and for the arbuscular mycorrhizal community in c) soils and d) roots. Diversity metrics were obtained from OTUs of both fungi and arbuscular mycorrhizal fungi. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

characteristics, particularly texture, were also found between the contrasting systems investigated here. Fertile soils with higher clay content are usually used as arable land, whereas grasslands historically have been maintained on less fertile soils or on areas that are more difficult to access. Thus, biotic differences between the systems paralleled differences in soil characteristics. However, this is not necessarily a confounding factor but rather an inherent property of grasslands in this landscape context that appears to support unique fungal taxa.

4.2. Diversity and abundance of fungi and AMF in grasslands vs leys

The lower fungal and AMF diversity in leys compared to grasslands suggest that certain management practices in leys may be affecting a subset of these communities. Common agricultural practices are known to decrease diversity of several trophic groups and promote homogenization of soil communities (Hartmann et al., 2014; Gámez-Virués et al., 2015; Gossner et al., 2016; Banerjee et al., 2024). This includes practices

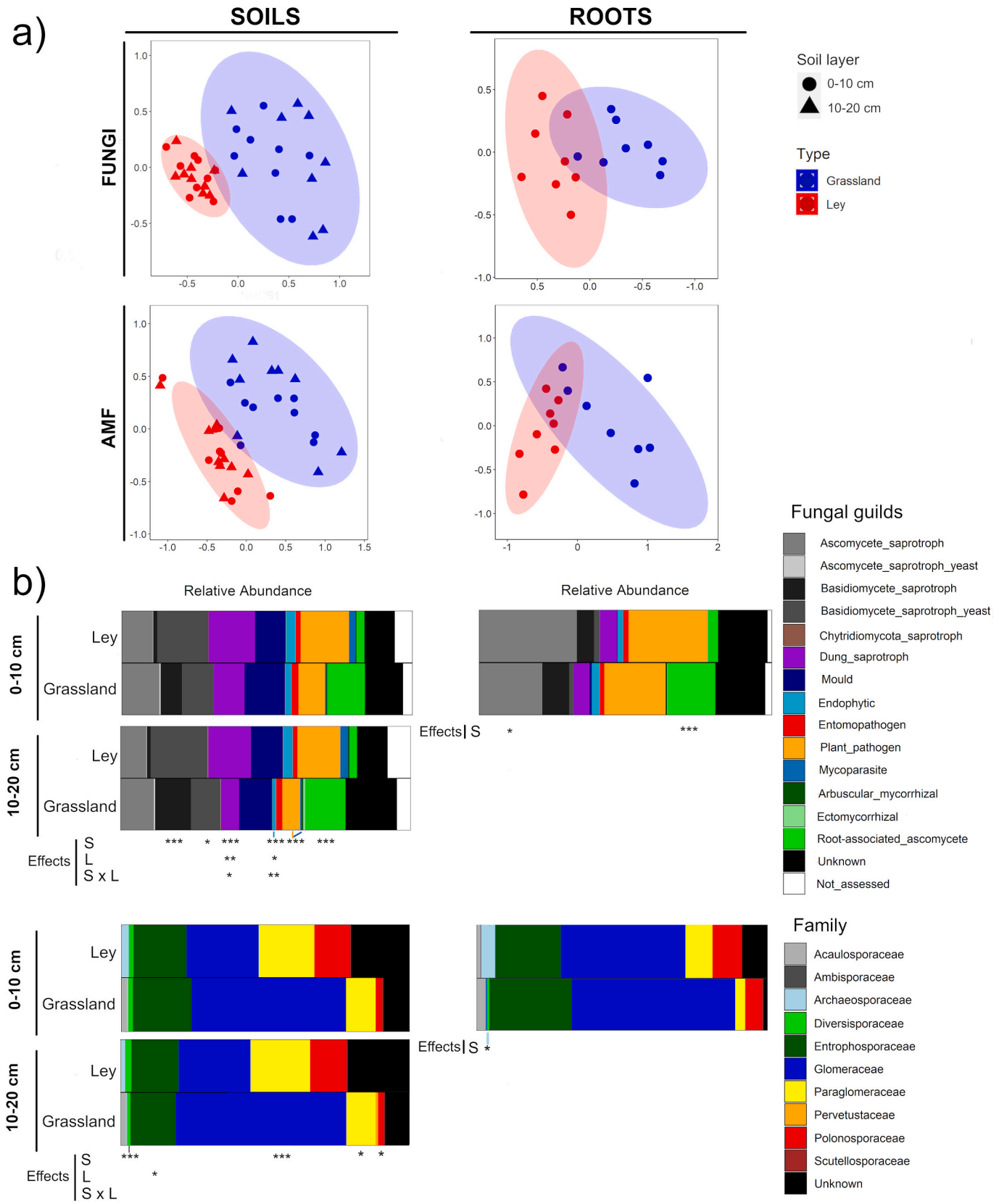

Fig. 3. a) Compositional and b) functional differences in fungal and arbuscular mycorrhizal communities between grasslands and leys in soils (left panels) and roots (right panels). In b) relative abundance of each fungal guild are shown, with significant effects indicated for system type (S), soil layer (L) and their interactions. Not_assessed guilds correspond to low-abundant taxa. *P*-values are indicated as: * = <0.05; ** = <0.01; *** = <0.001. Compositions are based on OTUs for both fungi and arbuscular mycorrhizal fungi.

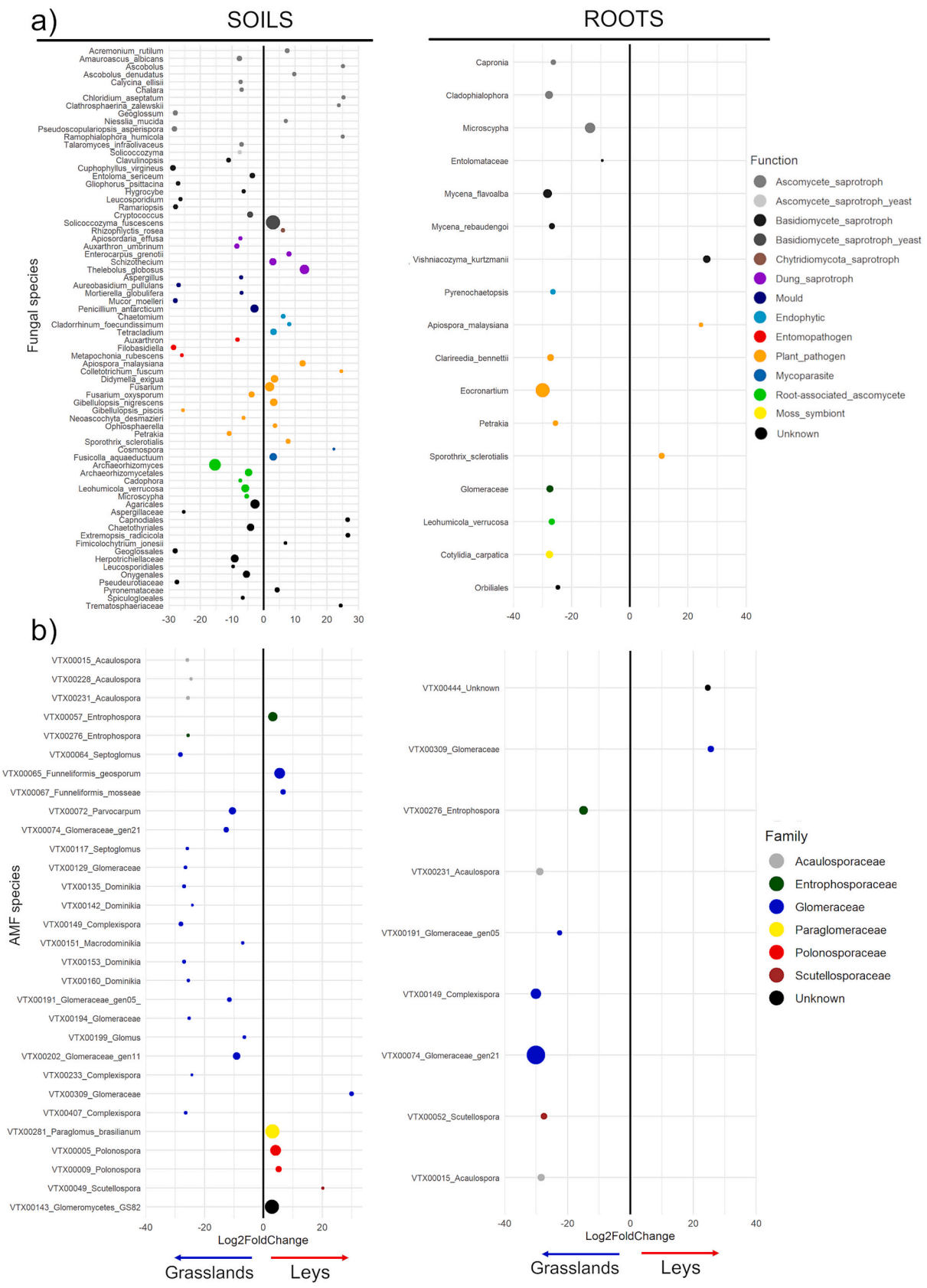

Fig. 4. Fungal (a) and arbuscular mycorrhizal fungal (b) species with a significant differential abundance between grassland and lay soils (left panels) and roots (right panels). Negative Log2 fold change values indicate higher abundance in grasslands (blue arrow) while positive values indicate higher abundances in leys (red arrow). Species are based on Species hypothesis for fungi and Virtual taxa for AMF, and coloured according to the fungal guild and family, for fungi and arbuscular mycorrhizal fungi, respectively. Dot size corresponds to the average relative abundance of the taxa. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

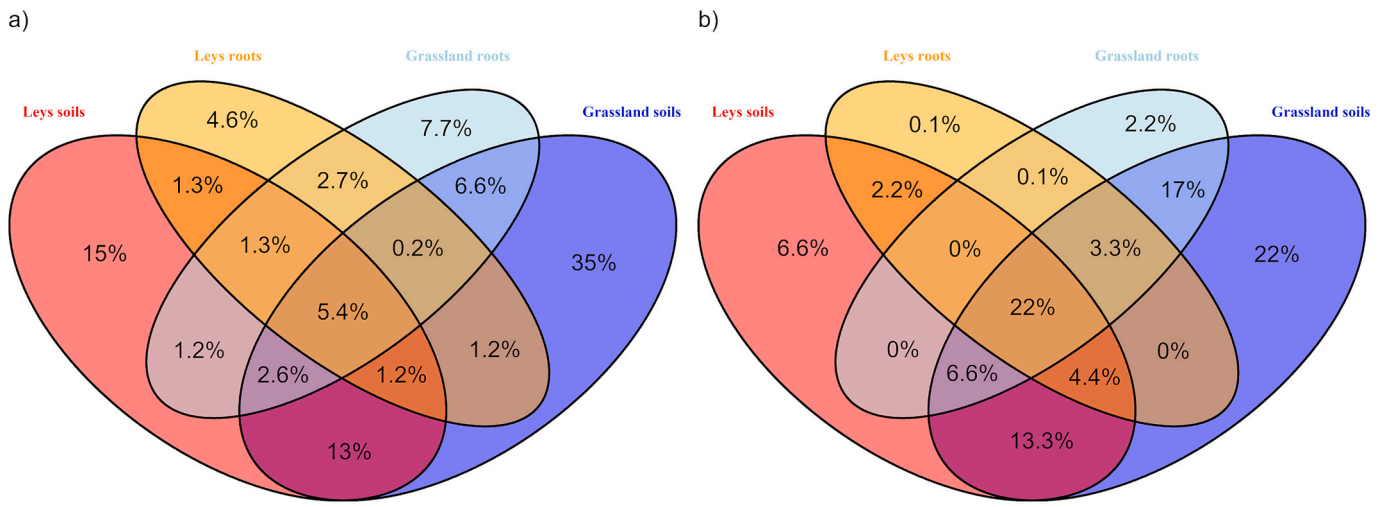

Fig. 5. Venn diagram showing the percentage of unique and shared species in grasslands and leys and in roots and soils for a) fungi and b) AMF.

like tillage, use of pesticides (e.g. for AMF fungi, Riedo et al., 2021) and N fertilization rates (Leff et al., 2015; Wang et al., 2018) and organic vs conventional farming (Hartmann et al., 2014; Peltoniemi et al., 2024, 2021). Fungal communities and diversity in grasslands have also been shown to differ in relation to soil fertility (Chen et al., 2020) and vegetation types (Guasconi et al., 2023). Although all the grasslands in this study were at least 150 years old, differences in their historical use, soil type, and more recent management likely contributed to variation among these, which could in part explain the higher heterogeneity across grasslands than across leys (Gossner et al., 2016). Their long history of no tilling and fertilization may have also contributed to greater heterogeneity in their biological communities, as further divergences in soil characteristics and biotic communities over long timescales could increase diversity at the landscape level (Tscharntke et al., 2012).

4.3. Taxonomical and functional differences of fungi and AMF between grasslands and leys

The composition of both total fungal and AMF communities differed between leys and grasslands, and consistent patterns remained when aggregating species abundances at guild level. We observed an overall higher relative abundance of putative pathogens in leys, which could be due to reoccurring monocultures in the rotations, which may promote pathogen accumulation (Wang et al., 2023). Despite that primary crops were followed by ley establishment in our sites, the fields may have accumulated pathogens over time. By contrast, higher plant diversity in grasslands could have a dilution effect on hosts susceptible to specific pathogens. A higher dominance of root-associated ascomycetes and saprotrophic basidiomycetes were instead observed in grasslands. Plants in grasslands may thus rely more on root-associated fungi or endophytes, than plants in leys, for uptake and assimilation of nutrients. This could potentially promote a more conservative N cycling mode with ecosystem N retention, as indicated by the lower $\delta^{15}N$ signatures in grasslands than in leys. Putative dung saprotrophs were also more abundant in leys, which could be the result of manure addition or more intensive grazing in these fields (Richter et al., 2024). Dung saprotrophs are copiotrophs that benefit from easily available C and inorganic N. By contrast, the grasslands contained more saprotrophic basidiomycetes, which are potentially better equipped for decomposing complex organic compounds (Floudas et al., 2012).

4.4. Shared species between compartments and associated to each system type

Several fungal taxa were significantly associated with either grasslands or leys. For soils, all the root-associated fungi and basidiomycete saprotrophs with significant associations were disproportionally found in grasslands. These taxa mainly belonged to *Archaeorhizomycetes*, but also dark septate endophytes such as *Cadophora* sp. and *Leohumicola verrucosa*, the latter also associated to grassland roots. These results are also supported by previous observations of increasing abundance of Helotiales species under organic vs conventional farming in boreal crops (Peltoniemi et al., 2024). Recently, *Cadophora orchidicola* was found to produce metabolites that inhibit pathogenic activity (Wang et al., 2019). *Leohumicola verrucosa* is a known ericoid mycorrhizal fungus but could have a more versatile ecology, as suggested by its presence in several grasslands without ericoid plants here. The basidiomycete saprotrophs associated to grasslands included *Hygrocybe* spp., *Cuphophyllus* sp., *Gliophorus* sp. and *Entoloma sericeum*, most of them considered of a particular conservational interest and often vulnerable species (Griffith et al., 2013). Several other *Hygrocybe* species were found only in grasslands, but did not arise as significantly associated to grasslands because of their low occurrences. Investigations into the ecological role of these species would shed light on the functioning of grassland soils and how these species contribute.

AMF species belonging to the genus *Dominikia* and *Acaulosporaceae* were also found to be indicators of grasslands, while species from other families, such as *Polonosporaceae* and *Paraglomeraceae*, were relatively more abundant in leys. A recent review indicates that the preference of certain AMF groups for either less or more disturbed soils is not supported by empirical evidence (Marro et al., 2022), although our study suggests that several *Glomus* species were more competitive in the less disturbed grasslands. What we found, instead, is a pattern of more numerous but more rare AMF species associated to grassland soils. This could be due to the presence of certain specialists in grasslands with narrow ecological niches, although such specialization seems to occur more at ecological groups level rather than at the individual species (Davison et al., 2011). It could also be that higher habitat heterogeneity among grasslands compared to among leys supports more disparate and rare communities, as indicated by the higher beta diversity across grasslands than across leys. By contrast, the higher disturbance levels in leys may favour fewer dominant disturbance-adapted AMF taxa, resulting in the lower diversity in young leys (Banerjee et al., 2019, 2024). In our Nordic grasslands a large fraction of nutrients may be locked up in organic forms, from which AMF have limited capability to mobilize nutrients (Read and Perez-Moreno, 2003; Smith and Read,

2008). However, AMF may still increase plant nutrient access through better competition for mineral nutrients released by saprotrophic decomposition and mineralization. The simultaneous higher abundance of root-associated ascomycetes, however, suggests that plants also rely more on other types of root associations; dark septate endophytes may have better competitive capacity to colonize roots than AMF in high latitudes (i.e. the sun-worshipper hypothesis proposed by Veresoglou et al., 2019). An overall higher AMF diversity in grasslands than in arable fields has been reported before (Oehl et al., 2004; Verbruggen et al., 2010), and our study points in the same direction, however it remains to be investigated whether Northern grasslands may be less suitable for certain AMF species and families.

4.5. Implications for soil functioning in grasslands and leys

Our results also suggest potential contrasting fungal-driven soil processes of leys and grasslands. In grasslands, larger root biomass and higher abundances of certain fungal groups may support plant nutrient uptake from surrounding soil via root symbionts and dark septate endophytes, potentially facilitated by unique species of basidiomycete decomposers. The larger proportions of unique species found in both grassland roots and soils suggest that fungal species have the potential to mediate a more diverse range of plant-soil interactions in grasslands than in leys. Several of the species uniquely found in grasslands were saprotrophic ascomycetes and basidiomycetes, and several species belonging to the genus *Mycena* were found exclusively in both roots and soils in grassland. Although *Mycena* is a widespread saprotrophic fungal genus, this finding aligns with recent studies showing that *Mycena* species can be found in host roots, potentially either forming mutualistic associations or accelerating root decomposition (Harder et al., 2023).

5. Conclusions

Protection of grasslands is important, not only for conservation of vascular plants and other aboveground organisms but also for the conservation of belowground fungi, given the large community of unique fungal taxa in grasslands for which we only know a fraction in detail. We highlight the importance of profiling unique belowground communities in semi-natural grasslands further to i) identify habitats supporting the accumulation and survival of rare species and species with conservational needs, and to ii) investigate which habitat management practices are needed to preserve these belowground communities and species. Finally, maintenance of grasslands by grazers, and management activities that minimize soil disturbances and avoid the use of fertilizers are likely to promote conservation of rare species.

CRediT authorship contribution statement

Carles Castaño: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anders Glimskär:** Writing – review & editing, Data curation, Conceptualization. **Sara Hallin:** Writing – review & editing, Resources, Methodology, Conceptualization. **Nadia I. Maaroufi:** Writing – review & editing, Conceptualization. **Helle Skånes:** Writing – review & editing, Data curation, Conceptualization. **Astrid Taylor:** Conceptualization, Writing – review & editing. **Karina E. Clemmensen:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2025.106233.

Data availability

Experimental design, community data and associated data of this study can be found in Mendeley Data, DOI: doi: 10.17632/5b88jb43hn.2.

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