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RESEARCH ARTICLE

Diversity, root tip vitality and soil-driven variations of Ectomycorrhizal communities in *Pinus cembra* forests from alpine treelines

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Abstract

Background and aims Swiss stone pine (*Pinus cembra*) is dominant tree species in the treeline ecotone of the European Alps. Ectomycorrhizal (EM) fungi are often used in treeline reestablishment with *Pinus cembra*, yet our understanding of natural EM communities is limited.

Methods In three alpine regions of the Austrian Alps on both silicate and calcareous bedrocks we determined the EM communities on roots of *Pinus cembra* and related these to soil chemical properties.

Results The taxa composition of the EM communities was similar for the different sites, but the structure in terms of abundance varied greatly. The community of the calcareous sites differed from the silicate sites, and correlated to the level of exchangeable Ca. Similarly, on the silicate sites the levels of total Al and Fe influenced the community structure.

Conclusion The composition of EM fungal communities of *Pinus cembra* is strongly influenced by soil factors.

Keywords Ectomycorrhizal fungi · Alpine timberline ecotone · Root tip vitality · Soil mineral composition · Exploration types

Introduction

High-altitude forests, such as those dominated by *Pinus cembra* in the European Alps, form the boundary between continuous forest cover and the treeless alpine zone, commonly referred to as the treeline

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ecotone (Zięba et al. 2019; Ulber et al. 2004). The alpine treeline ecotone is characterized by long, cold winters, extended periods of snow cover, and a short growing season (Casalegno et al. 2010). *Pinus cembra* is a relict species of the last glaciation and has a treeline distribution in European mountain ecosystems. *Pinus cembra* occurs on soils formed on both siliceous and carbonate bedrock (Ulber et al. 2004), which are common within the natural distribution of this species (Zieba et al. 2019). However, the ecology of *Pinus cembra* on these two bedrock is largely unknown. High-altitude forests are currently a focus of conservation because of losses through agricultural encroachment, but also through climate change effects at the treeline (Casalegno et al. 2010, Wieser et al. 2019). Measures to restore treelines include the use of ectomycorrhizal inoculated seedlings (Ulber et al. 2004; Rainer et al. 2015). Previous studies on mycorrhizas of *Pinus cembra* have mainly focused on nursery plants or experimental sites, and provided important early insights into suitable mycorrhizal partners and substrates (Göbl 1970; Keller 1989; Göbl and Ladurner 2000; Bacher et al. 2010; Rainer et al. 2015), but comprehensive community-level data from natural high-altitude forests remain scarce. Recent work of other high-altitude conifers, such as *Larix decidua*, has shown that ectomycorrhizal communities are diverse and dynamic (Mandolini et al. 2025), and that soil fungal communities in *Pinus cembra* forests exhibit distinct patterns under snow-free and snow-covered conditions (Probst et al. 2024). However, ectomycorrhizal communities of *Pinus cembra* roots across contrasting calcareous and silicate bedrock have not yet been systematically characterized.

Ectomycorrhizal communities are composed of broad, intermediate and narrow range fungi (Lang et al. 2011). *Pinus cembra* together with other pine species of the subgenus *Strobus*, forms a host specific narrow range symbiosis with the ectomycorrhizal genus *Suillus*. In particular, *Pinus cembra* forms ectomycorrhizas with the taxa *Suillus plorans*, *S. placidus,* and *S. sibiricus* (Rainer et al. 2015; Mandolini et al. 2022). *Suillus* ectomycorrhizas are initially coralloid, with a large number of ectomycorrhizal tips on an individual ectomycorrhizal unit, but develop into a single nodule-like structure (Göbl and Ladurner 2000; Rainer et al. 2015). Due to the specificity of the *Pinus cembra- Suillus* symbiosis, these *Suillus* species have been the focus of afforestation to re-establish the treeline (Keller 1996; Rainer et al. 2015), and have been shown to dominate the EM communities of planted seedlings (Keller 1996). The ectomycorrhizas of *Suillus* are long-distance exploration types (Peay et al. 2011) and form an extensive extramatrical mycelium. Long-distance exploration types have been shown to be more common in areas where tree root density is low, for example on the edges of tree islands (Peay et al. 2011). Tree islands are a common structure at the alpine treeline (Wang et al. 2017).

Although there is increasing recognition of the ecological importance of EM fungi in high elevation forests (Mühlmann and Peintner 2008), understanding of EM fungal community composition, and the environmental factors that influence the composition, remains incomplete. In one of the few investigations of natural stands of *Pinus cembra*, a similar EM taxa diversity was found for adult trees at four different high-altitude sites, but with a large variation in taxa composition of the EM communities between sites (Mandolini et al. 2025). In lowland forests, soil type is known to influence the structure of ectomycorrhizal communities (Pena et al. 2017). In *Fagus sylvatica*, in both adult trees (Pena et al. 2017) and seedlings (Leberecht et al. 2016), the EM community structure differed in calcareous soils compared to neutral soils. Similarly in tropical *Acacia*, Houles et al. (2018) showed differences in EM communitues between calcareous soils and ferrolitic and volcanic soils. However, the study was carried out on a low number of root tips, and the all of the calcareous sites were islands, which may have limited the number of EM species present. In one of the few investigations of high elevation sites, in *Picea abies,* EM communities were shown to differ between calcareous and silicate bedrocks (Scattolin et al. 2008). Scattolin et al. (2008) showed that EM community composition was related to pH of the soil and also the exposition of the slope. In Artic ecosystems, the availability of Ca, Mg and N were specifically the most important factors affecting the EM community composition (Timling et al. 2012). Soil temperature is also a factor which influences tree growth at the treeline (Wieser et al. 2019), and varies with exposition. In high elevation *Pinus pumila* forests, EM fungal communities were shaped primarily by soil temperature (Koizumi and Nara 2020). In contrast, short term manipulation of soil temperature in *Pinus cembra* did not result in

significant changes community composition (Rainer et al. 2015). In *Pinus uncinata* growing at high elevation, soil warming changed the structure of the ectomycorrhizal community, but this change was however due to warming-induced changes in soil N availability rather than a direct effect of temperature (Solly et al. 2017). However, many of the above studies are based on analysis of a relatively low number of ectomycorrhizal root tips, which results in an over estimation of dominant taxa and does not sample rare taxa (Lang et al. 2011; Otgonsuren et al. 2020).

In the present study, the EM fungal communities in *Pinus cembra* at the treeline ecotone in the Austrian Alps growing on soils of both calcareous and silicate bedrocks were investigated. The objectives were: (i) to determine the EM fungal community structure across different isolated mountain ranges, (ii) to elucidate how soil parameters affect EM fungal abundance and taxa, (iii) to assess potential differences in vitality of the EM community based on vital and sub-vital ectomycorrhizal root tips. Our investigation focused on *Pinus cembra* growing on calcareous and silicate sites, in which we have used a low number of sites, but a very high sampling effort to gain the best estimate of EM community structure.

Materials and methods

Study site description

Three different regions of Austrian Alps, the Zirbitzkogel, Stoderzinken and Nockberge (Table 1), were chosen to carry out the study on the ectomycorrhizal (EM) fungal communities of *Pinus cembra* Du Tour. The sites situated between 1800 and 1950 m in elevation, represent the transitional zone between the timberline and the treeline. This zone is dominated by *Pinus cembra* which forms either isolated tree islands or an abrupt treeline in certain areas (Harsch et al. 2009; Xu et al. 2020). The parent material of the Zirbitzkogel and Nockberge sites is dominated by schist, gneisse and granites, typical for the central range of the Austrian Alps (Neuwinger 1972, 1980), whereas Stoderzinken is situated at the southern edge of the Northern limestone Alps. Mean annual temperature was similar among the sites, whereas mean annual precipitation was higher at Stoderzinken compared to the other two locations. The slope varies considerably at all sites. The sampled areas were on the moderate 10 to 28 degree slopes. The soils at the sites are classified as Leptosols, with the top soil horizon mainly either and Oh or Ah layer often covered by a layer of undecomposed *Pinus cembra* needles.

Sample collection

Root and soil samples were collected in August 2022. At each of the three sites Zirbitzkogel, Stoderzinken and Nockberge, five plots were randomly selected within mature tree stands of *Pinus cembra*. These natural stands are composed of dominant and sub-dominant trees with tree heights between 18 and 25 m. The plots were in a continuous forest area, and were often large tree islands of 5 to 10 trees on rocky outcrops. To ensure independence, the plots were a minimum of 50 m apart and up to 500 m apart depending upon the topography and the occurrence of patches where samples could be taken. The majority of the plots were flat areas in a steeply sloping rocky terrain, so that the plots were separated not only by distance but also by elevation and physical rock barriers. The

Table 1 Characteristics of the natural *Pinus cembra* forests at the three sites studied at the timberline ecotone of Austrian Alps

Sites	Zirbitzkogel	Stoderzinken	Nockberge
Geographic coordinates	47°4′40″ N 14°35′1″ E	47°27′33″ N 13°49′17″ E	46°53′33″ N 13°48′46″ E
Elevation (m)	1835–1870	1830–1910	1770–1790
Annual temperature (°C)	5.3	5.8	4.7
Annual precipitation (mm)	1621	2025	1648
Slope (°)	10	28	20
Vegetation cover (%)	80	80	60
Soil Geology	Leptosols Mica slates Gneiss	Leptosols Limestone	Leptosols Mica slates Gneiss

samples were taken using a nested design. To account for variability within a plot, at each plot five soil cores were collected with a soil coring tool (8 cm diameter) to a soil depth of 10 cm. The soil cores were a minimum of 1 m apart and within a 5 m diameter circle. The cores were later combined for soil analysis. Prior to sampling, the needle litter on the surface layer of the soil was removed. The cores were placed into plastic bags and stored in a cool box, then at 4 °C in a laboratory storage facility.

Sample preparation and morphotyping

From each core and soil depth, roots were carefully removed from the soil, the adhering soil removed, and carefully washed over a 1 mm mesh sieve. A fine paint brush was used to remove materials tightly adhering to the ectomycorrhizas, ensuring minimal disturbance. The ectomycorrhizas were sorted into morphotypes using a AxiCam ERc5s microscope. The characterization of EM was based on morphological characteristics (branching pattern, shape and color, mantel characteristics and emanating hyphae characteristics) based on the classification system of Agerer (Agerer 2001), and using the Information System for Characterization and Determination of Ectomycorrhizae (DEEMY) database (http://www.deemy.de/) (Agerer and Rambold 2004).

The abundance of the different morphotypes was characterized based on both the number of EM fungal units and number of EM fungal taxa root tips (Fig. 1). The EM root tips were further categorized as vital tips and sub-vital tips based on external features of the ectomycorrhizas (Cudlín and Chmelíková 1999). Vital tips were turgid and undamaged, subvital tips were composed of non-turgid darkened tips and fractured or incomplete root tips (Fig. 1) (Horton et al. 2013). The relative abundance of morphotypes was based on the number of vital root tips or units, the abundance of vital or sub-vital tips was based on the sum of all tips (vital plus sub-vital). A unit was defined as a number of root tips on a single attachment point to the fine root (Fig. 1).

The EM exploration types associated with *Pinus cembra* were categorized into contact exploration, short-distance exploration, medium-distance exploration and long- distance exploration based on images taken using the AxiCam ERc5s microscope. Each morphotype was separately stored in Eppendorf-tubes

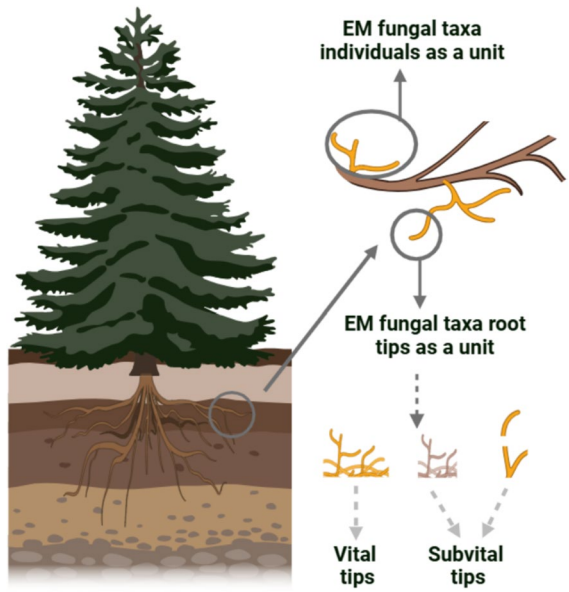


Fig. 1 Illustration of EM fungi unit concept; EM fungal taxa individuals and EM fungal taxa root tips as units, respectively. Drawn using https://app.biorender.com/gallery

(2ml) containing deionized water at −20°C until DNA extraction. The number of root tips varied from one to ten, depending on the abundance of the morphotype. The final identification to genus or species level (where possible) was carried out by amplicon sequencing and identification.

In total 9414, 17,044 and 12,429 ectomycorrhizal root tips were sorted into morphotypes for Zirbitzkogel, Stoderzinken and Nockberge, respectively.

Molecular identification

DNA was isolated using the MagAttract PowerSoil DNA Kit (Qiagen) in a 96 well microplate, following the manufacturer's protocol with slight modifications. For each extraction, we used single ectomycorrhizal root tips representing individual morphotypes; for coralloid morphotypes (e.g. *Suillus plorans*), a small nodule fragment consisting of several tips of the same morphotype was used. Owing to the limited sample volume in each well, the quantities of all buffers were halved. The automated procedures were carried out using a Hamilton Liquid Handling station. The extracted DNA was then utilized for amplifying fungal-specific ITS regions (Amenyogbe et al. 2021).

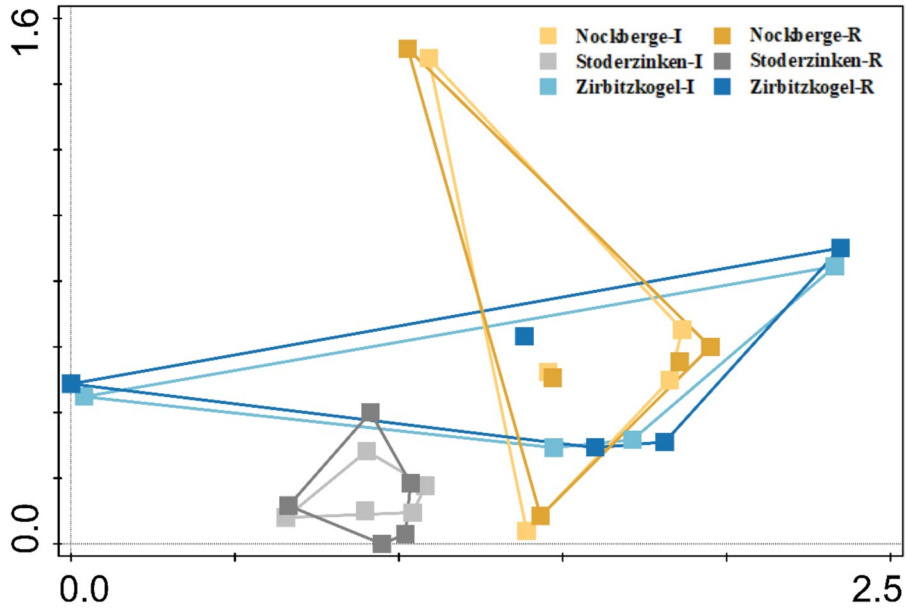


Fig. 2 Sample plots of detrended correspondence analyses (DCA) of EM fungal communities of *Pinus cembra* at Zirbitzkogel, Stoderzinken and Nockberge. I, Taxa abundance based taxa individuals; R, Taxa abundance based root tips

A two-step method was used for the amplification process. Initially, two fragments were amplified: one encompassing a portion of the SSU, the ITS region, and a part of the LSU using a fungal-specific primer pair (FungiQuant-F and TW13) (White 2008; Liu et al. 2012), and the other targeting Basidiomycota with the primer pair Basid2R + and LB-WT (Lynch and Thorn 2006; Tedersoo et al. 2008). Subsequently, a secondary amplification was conducted on the pre-amplification products using the ITS1F and ITS4 fungal-specific primers (Gardes and Bruns 1993). GoTaq G2 Green Master Mix (Promega) was utilized for all amplification steps, and the annealing temperature was maintained at 54°C.

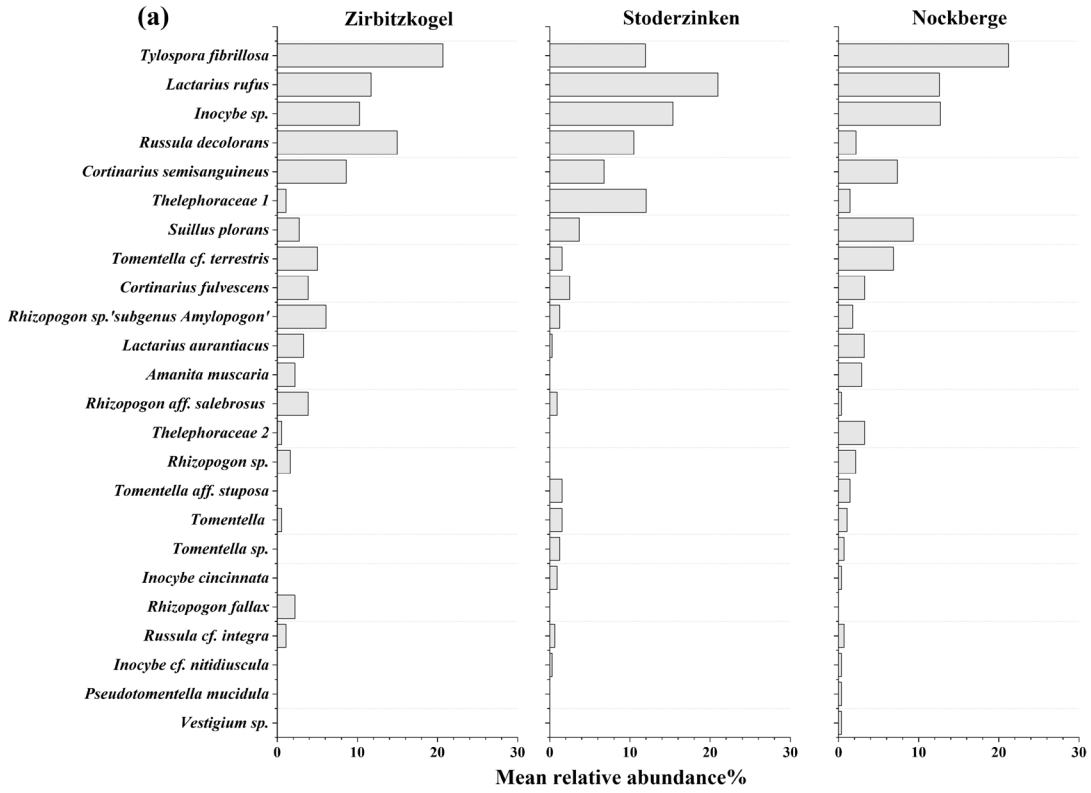
All products from second amplification were sequenced from both ends and forward and reverse reads were subsequently assembled. The molecular identification process involved BLASTn (https://blast.ncbi.nlm.nih.gov) searches in the NCBI (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi) database (Frank et al. 2008). Priority was given to matches with sequences from isolates in acknowledged culture collections. It is important to note that for the molecular identification only the ITS region was used, which does not offer adequate species resolution in all taxa. Therefore, the results must be considered sensu lato.

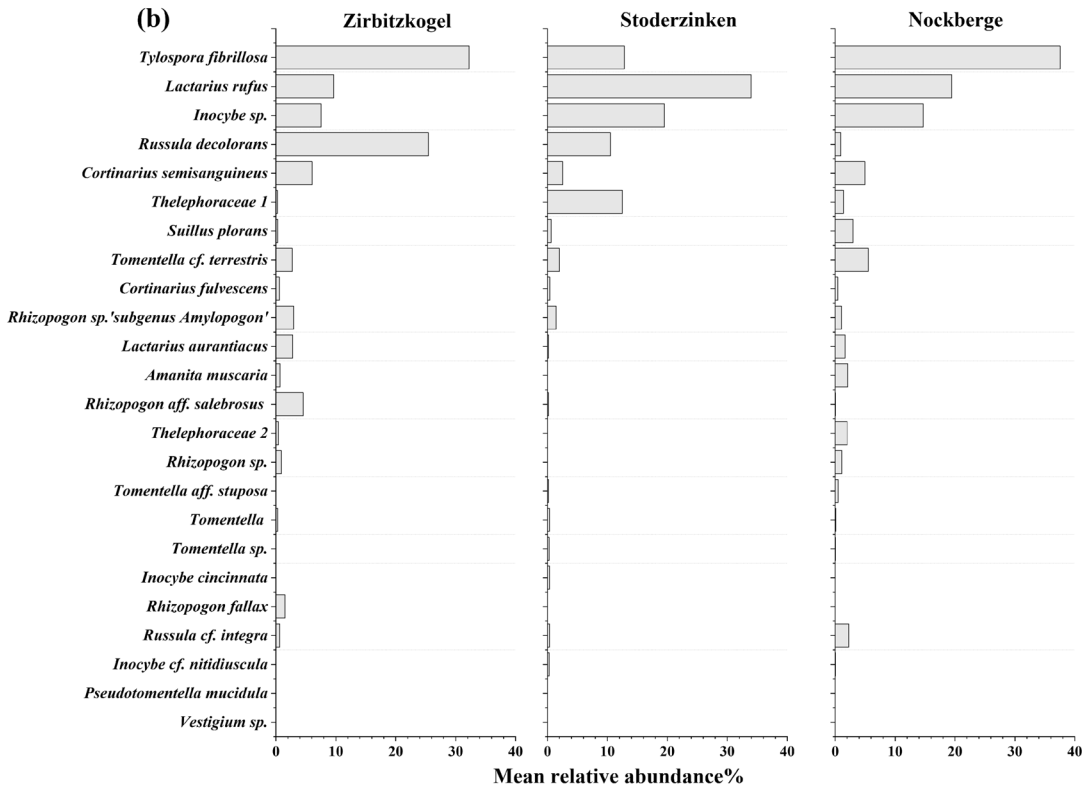
Soil chemical analysis

The soil remaining after the roots were removed and the additional soil removed from the fine roots were combined, and was passed through a 2 mm mesh sieve. Soil from the single cores were combined to give a bulk sample for each of the 5 plots. Using the fresh soil, pH was measured using a pH electrode of 1:3 (w/v soil: extractant) soil suspension in distilled water (Feng et al. 2022). The remaining soil was dried at 105 °C. Total C and total N were measured in a LECO TruSpec CN analyser (Leco, St. Joseph, USA). Extractable Al, Ca, Fe, K, Mg Mn and Na

Table 2 Richness (EM fungal taxa richness) and alpha diversity indices for three mountains

Sites	Richness (n)	Shannon (H′)	Evenness (J′)	Dominance (λ)	Margalef (d)
Zirbitzkogel	18	2.9 ± 0.33a	1.0 ± 0.02a	0.06 ± 0.01a	6.8 ± 1.01a
Stoderzinken	18	2.6 ± 0.47b	0.7 ± 0.04b	0.10 ± 0.01b	5.2 ± 0.97b
Nockberge	23	3.0 ± 0.20a	0.8 ± 0.05b	0.07 ± 0.01a	7.3 ± 0.71a


(a)
Zirbitzkogel
Stoderzinken
Nockberge
Tylospora fibrillosa
Lactarius rufus
Inocybe sp.
Russula decolorans
Cortinarius semisanguineus
Thelephoraceae 1
Suillus plorans
Tomentella cf. terrestris
Cortinarius fulvescens
Rhizopogon sp.'subgenus Amylopogon'
Lactarius aurantiacus
Amanita muscaria
Rhizopogon aff. salebrosus
Thelephoraceae 2
Rhizopogon sp.
Tomentella aff. stuposa
Tomentella
Tomentella sp.
Inocybe cincinnata
Rhizopogon fallax
Russula cf. integra
Inocybe cf. nitidiuscula
Pseudotomentella mucidula
Vestigium sp.
0 10 20 30
Mean relative abundance%


(b)
Zirbitzkogel
Stoderzinken
Nockberge
Tylospora fibrillosa
Lactarius rufus
Inocybe sp.
Russula decolorans
Cortinarius semisanguineus
Thelephoraceae 1
Suillus plorans
Tomentella cf. terrestris
Cortinarius fulvescens
Rhizopogon sp.'subgenus Amylopogon'
Lactarius aurantiacus
Amanita muscaria
Rhizopogon aff. salebrosus
Thelephoraceae 2
Rhizopogon sp.
Tomentella aff. stuposa
Tomentella
Tomentella sp.
Inocybe cincinnata
Rhizopogon fallax
Russula cf. integra
Inocybe cf. nitidiuscula
Pseudotomentella mucidula
Vestigium sp.
0 10 20 30 40
Mean relative abundance%

◂**Fig. 3** Abundance of EM fungal taxa individuals (I) and EM fungal taxa root tips (R) were compared among three different sites (Zirbitzkogel, Stoderzinken and Nockberge) within *Pinus cembra* trees. **a**: Taxa abundance based EM fungal taxa individuals; **b**: Taxa abundance based EM fungal taxa root tips

were determined in a 1:3 (w/v soil: extractant) extraction in 0.1M $BaCl_2$. Total contents of these elements were extracted by acid digestion using concentrated HCl/HNO_3 (3:1 ratio) using a CEM Mars 6 Microwave system. Exchangeable P was extracted in 0.5M $NaHCO_3$ solution. All elements were determined by ICP on a Perkin Elmer 8300 ICP-OES instrument.

Statistical analyses

Data collected from the field and laboratory analyses were subjected to statistical analysis using Origin software and Canoco (version 5.0; Ter Braak and Šmilauer 2012). The structure of the EM fungal community, based on the abundance of individual EM fungal taxa and root tips across all samples, was evaluated through detrended correspondence (DCA) analysis. The community structure of EM fungal taxa associated with vital and sub-vital tips was analyzed using non-metric multidimensional scaling (NMDS), based on the Adonis test and Bray–Curtis distance matrix, to assess the effects of experimental factor (locations) (Oksanen 2011). The EM fungal diversity was expressed at the species level using taxa richness (S), Shannon diversity index (H′), Pielou evenness (J′), Simpson′s dominant index (λ), and Margalef index (d) (Du et al. 2020; da Silva et al. 2012). Richness (S) was obtained by counting the number of EM fungal taxa at each site. The Shannon diversity index was calculated according to the equation $H' = -\Sigma$ (Pi ln [Pi]), where Pi = ni/N, ni = number of individuals of species i, N = total number of individuals all species. Pielou evenness was obtained by the equation J′ = H′/Log (S), where H′ is the value obtained by the Shannon index and S is the total number of species. Simpson′s dominant index (λ) was calculated according to the equation $\lambda = \Sigma$ (i = 1 to S) Pi^2, Pi = proportion of individuals of species i relative to total individuals. The Margalef index was calculated by the equation d = S − 1/LogN. These metrics were calculated based on the overall relative abundances of taxa for each site.

Relative occurrence frequencies were calculated by counting the number of times a taxon appeared among individuals within the same sample across the three sites. Frequencies were reported as percentages. In the heatmap, clustering analysis for the categorical factors was conducted using Euclidean distances (Mandolini et al. 2024). Redundancy analysis (RDA), and correlation analysis were performed to investigate the relationships between different soil parameters and the abundance of EM fungi.

Results

Diversity and composition of EM fungal units across different mountains

The detrended correspondence analysis (DCA) plot showed differences in the composition of EM fungal communities associated with *Pinus cembra* between sites (Fig. 2). For each site, a similar pattern was found whether the abundance was based on EM fungal taxa individuals or root tips. The EM fungal communities at the two gneiss sites Zirbitzkogel and Nockberge overlapped, whereas the community at the limestone site Stoderzinken was separated. Zirbitzkogel and Nockberge exhibited a broad spread, suggesting greater variability and heterogeneity in fungal taxa at this site between sampling points. In contrast, Stoderzinken showed more compact clustering, indicating a more homogeneous fungal composition between sampling points.

Based on a number of ecological indices, Stoderzinken exhibited a strong difference to the other sites (Table 2). The highest number of taxa were found at Nockberge and the lowest at Zirbitzkogel. Rarefraction analysis confirmed this relationship at similar sampling efforts (Fig. S1), and confirmed that with the high sampling effort the majority of taxa had been detected. Using the Margalef index which also takes into account the sampling effort, again higher values of taxa richness were shown for Zirbitzkogel and Nockberge compared to Stoderzinken. The EM community at Stoderzinken showed a lower value for evenness and thus a higher value for dominance compared to the other two sites, that had similar values. The higher dominance index at Stoderzinken suggests a prevalence of specific taxa. Nockberge had the highest species richness (24 species) and the highest Margalef's richness index (7.28 ± 0.71).

The data are the means ± standard error. Values followed by different letters for each alpha diversity

indices are significantly different ($p < 0.05$) according to Tukey's test.

Different taxa dominate EM fungal community in each mountain site

In total, 785 samples from EM fungal taxa individuals (I) and 38,887 samples from EM fungal taxa root tips (R) were analyzed. The community structure based on root tips (Fig. 3b) showed a much higher dominance of a few taxa, compared to when the community structure was based on individual units (Fig. 3a). This is as the number of root tips of the abundant taxa *Lactarius rufus* and *Tylospora fibrillosa* have up to 80 EM tips per EM fungal taxa individual. Whereas taxa with lower abundance exhibit root tip numbers ranging from 1 to 7 per EM fungal taxa individual. Irrespective of whether the community structure is based on tips or individuals, *Tylospora fibrillosa* and *Lactarius rufus* were the most abundant taxa at all sites. These two taxa also showed the highest occurrence frequencies across all sites, occurring in 80% or 100% of sampling points within a site (Fig. 4). At Nockberge and Zirbitzkogel, *Tylospora fibrillosa* was the most abundant taxon and had a 100% frequency. In contrast, at the calcareous Stoderzinken site the most abundant taxon was *Lactarius rufus*. At Zirbitzkogel, *Russula decolorans* was the second most abundant taxon, but was present only at lower abundance at the other silicate site Nockberge. *Suillus plorans* was present in low to medium abundance and all sites, with a frequency of 40–60%. The low abundance taxa occurred with both a high and low frequency. *Lactarius badiosanguineus* was low in abundance at all sites, but at Stoderzinken had an occurrence frequency greater than 80%. *Pseudotomentella mucidula* and *Vestigium sp*. were only detected in Nockberge, appearing with low abundance and had a low occurrence frequency, while *Rhizopogon fallax* was only found in Zirbitzkogel. The taxa *Amanita muscaria*, *Thelephoraceae 2*, and *Rhizopogon sp.*, did not occur at Stoderzinken, but were found at the other sites (Fig. 3, 4).

When the taxa were divided into exploration types (Fig. 5) the EM community at Stoderzinken had a higher relative proportion of contact exploration types compared to the communities at Zirbitzkogel and Nockberge. At Stoderzinken contact exploration types were the most common type in the EM community, whereas at both Zirbitzkogel and Nockberge short distance exploration types were the most common.

Community comparisons of EM fungal root tip types

The analysis of EM fungal taxa across different root tip categories (vital and sub-vital) showed that most taxa had a greater proportion of vital tips compared to sub-vital tips across all sites (Fig. 6). Notably, *Suillus plorans* had an overall low proportion of sub-vital tips, below 1% across all sites. Similarly, no sub-vital tips were observed in *Rhizopogon sp.*, *Pseudotomentella mucidula*, and *Vestigium sp.* In contrast, at Zirbitzkogel and Nockberge, *Thelephoraceae 2* had a high proportion of sub-vital tips, as well as *Inocybe sp.*, *Amanita muscaria*, *Russula cf. integra*, and *Tomentella.*

Relationships between abundances of EM fungal communities and soil parameters

In the 0 to 10 cm soil layer of the 3 sites (Table 3), the soil pH was significantly higher at Stoderzinken compared to Zirbitzkogel, but not to Nockberge. The Nockberge site was characterized by having significantly higher amounts of total Al, Fe and Mg compared to the other sites. But the higher total content did not result in higher exchangeable contents. At Stoderzinken the calcareous bedrock was reflected in higher contents of total and exchangeable Ca, and lower contents of exchangeable Al.

Exchangeable and total element concentrations are shown. Values followed by different letters within a row are significantly different at $P < 0.05$. Shown are means ± standard error. $N = 5$.

Redundancy analysis (RDA) was carried out within each EM fungal taxon using 20 soil chemical parameters to identify potential influences across the study regions (Fig. 7). The RDA revealed a strong correlation between soil nutrient variables and the abundance of EM fungal vital (Fig. 7a and 7b) and sub-vital tips (Fig. 7c and 7 d). For clarity the fungal taxa are presented as two groups. Each axis, RDA1 and RDA2, showed the proportion of variance explained, ranging from approximately 16% to 27% for RDA1 and around 12% to 17% for RDA2. In terms of relative abundance across EM fungal vital and sub-vital tips, *Russula decolorans* and *Thelephoraceae1* demonstrated significant positive

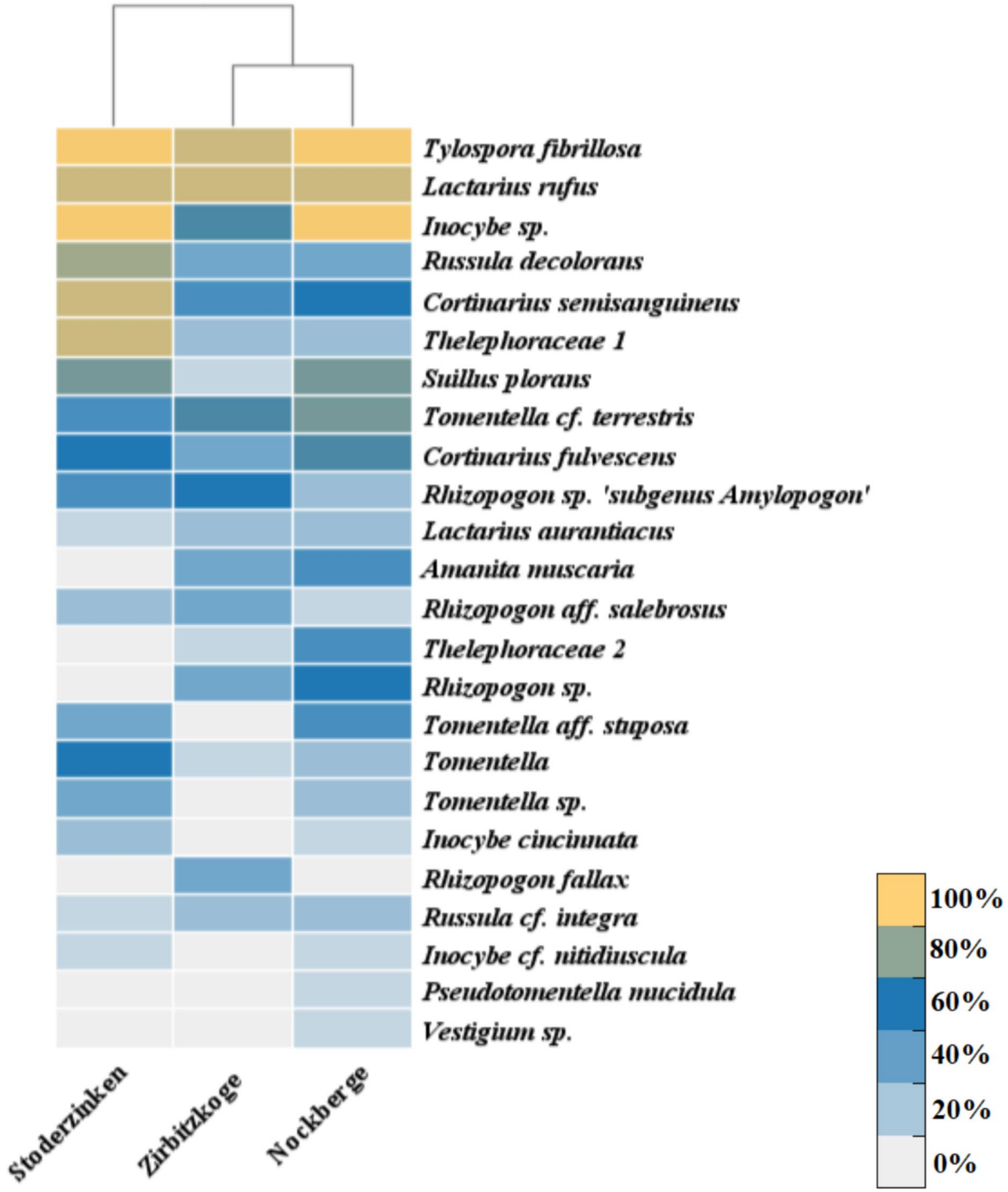


Fig. 4 Heatmap and accompanying cluster analysis (x axis) of the relative occurring frequency of EM fungal taxa across *Pinus cembra* of different sites (Zirbitzkogel, Stoderzinken and Nockberge). The occurrence frequency for each taxon is colored in the shades of yellow (high frequency) to blue (low frequency). The frequency percentage indicated how often each taxon was detected across replicates, spanning from 100% for taxa found in all replicates to 0% for those not detected in any replicate

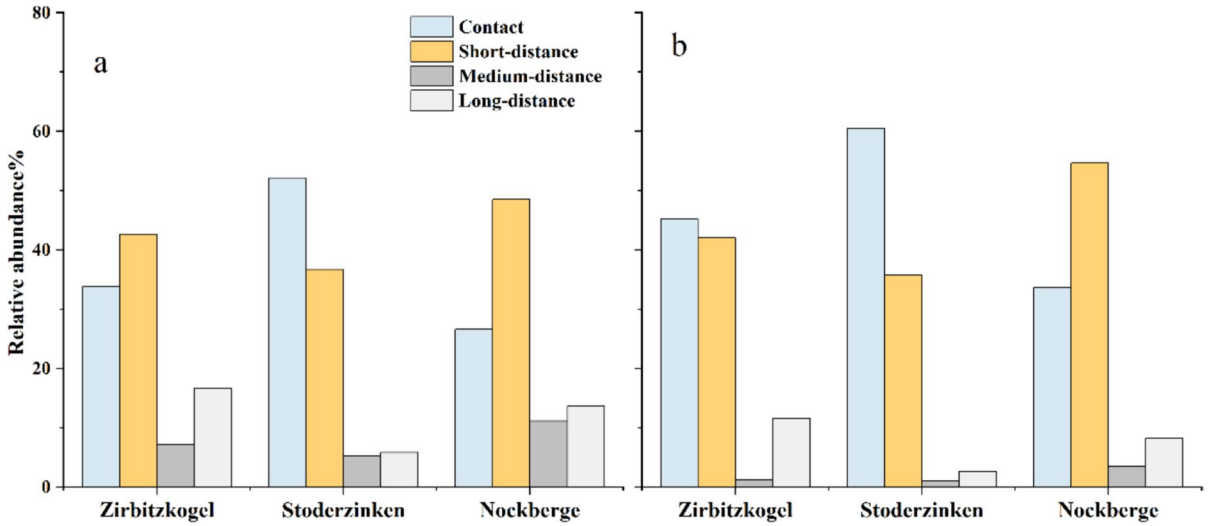


Fig. 5 Relative abundance of EM fungal exploration types in different sites. a, Taxa abundance based taxa individuals; b, Taxa abundance based vital tips

correlations with total C, N and S. *Amanita muscaria* showed a significant negative correlation with total C, N and S, but exhibited a positive correlation with total Na and P, and exchangeable Fe and Al. Additionally, pH, total and exchangeable Ca was significantly positively correlated with *Suillus plorans*, *Lactarius badiosanguineus*, and *Lactarius rufus*. *Cortinarius* genus displayed a notably stronger positive correlation with pH and exchangeable Mn in vital tips compared to sub-vital tips. Within vital tips, the relative abundance of *Rhizopogon sp.* was significantly negatively correlated with total C, N and S. For pH, total Ca, and exchangeable Mg, the relative abundance of *Rhizopogon aff. salebrosus* showed significant positive correlations within sub-vital tips, while no correlations were observed in vital tips. Conversely, the relative abundance of *Russula cf. integra* demonstrated significant positive correlations with these soil properties across both root tip types. Furthermore, low-abundance taxa such as *Pseudotomentella mucidula* and *Vestigium sp.* also exhibited significant positive correlations with pH, total Ca and exchangeable Mg.

RDA revealed that soil physicochemical parameters collectively explained 87.29% of the total explained variance in EM fungal exploration types based on taxa individuals and 87.28% based on vital tips, indicating a similar influence of soil properties on EM fungal exploration types in both analyses (Fig. 8). The three study sites exhibited distinct clustering patterns, suggesting that strong site-specific regulation of EM fungal exploration types by local soil conditions. The contact exploration type was closely associated with soil pH, and exchangeable Ca Mg, particularly at Stoderzinken. Short- and medium-distance exploration types showed a significant positive correlation with Fe and Al, predominantly clustering with the Zirbitzkogel and Nockberge. Long-distance exploration types were positively associated with exchangeable K and Na, as well as total C, N and S.

Discussion

Ectomycorrhizal community structure of natural treeline sites

In this study, the *Pinus cembra* roots tips showed nearly 100% mycorrhization, consistent with findings reported by Mandolini et al. (2022) and Rainer et al. (2015). The EM community was composed of between 18–23 EM fungal taxa per location, while Mandolini et al. (2022) documented in total 20 fungal taxa associated with mature *Pinus cembra* in South Tyrol across four sites. In our investigation, across the mountain sites there were differences in the diversity and composition of EM fungal communities associated with *Pinus cembra.* Although the

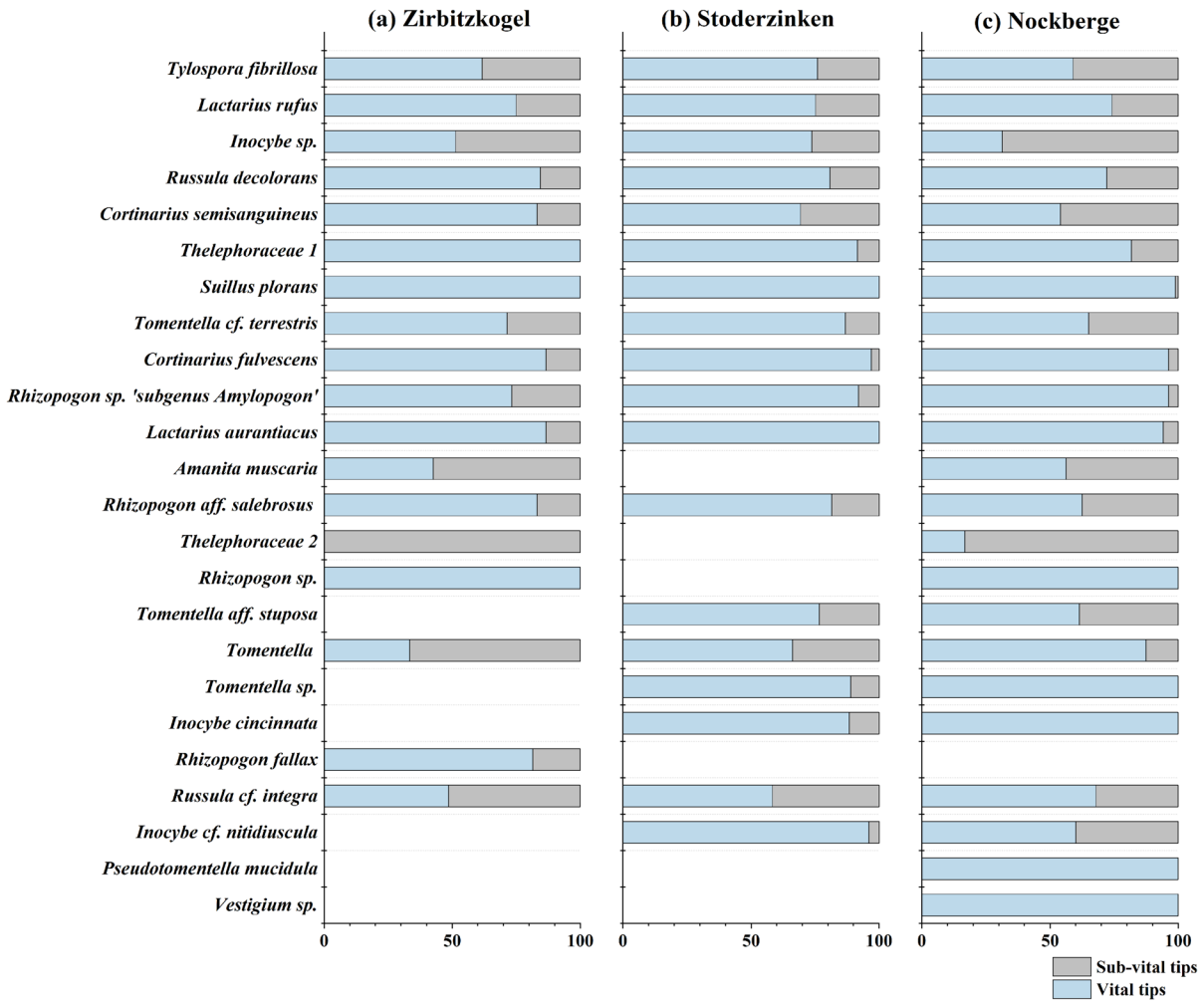


Fig. 6 Relative abundance of EM fungal taxa root tips categorized as vital and sub-vital tips in different sites

EM taxa composition was largely similar between sites, differences occurred in the abundance and frequency of individual EM taxa. The variation in taxa richness was most pronounced among the low-abundance taxa. Such differences can only be detected using a very high number of root tips as used in this study. However, as we carried out a single sampling, temporal variation has not been quantified. The rarefaction analysis (Fig. S1) indicated that most taxa had been detected, and hence the differences in taxa composition are real. Mandolini et al. (2022) showed much greater difference in EM communities of *Pinus cembra* from four sites in the southern Alps, also in highly abundant taxa. However, Mandolini et al. (2022) sampled a much lower number of root tips, hundreds rather than the thousands used in the current study, which may account for the differences between the studies. However, both studies showed a high dominance of *Russula decolorans*.

The relative abundance of a taxon differs for many taxa depending whether abundance is based on the number of root tips or the number of individual EM fungal units. This difference is due to morphological differences among fungal taxa (Agerer 1991, 2001). This is particularly the case for coralloid taxa such as *Suillus plorans* which produce tens of ectomycorrhizal tips per individual, which may exaggerate their apparent dominance when analyzing ectomycorrhizal tips alone. Although, *Suillus plorans* is the focus of considerable interest in treeline restoration (Rainer

Table 3 Soil chemical properties of the 0–10 cm soil layer at Zirbitzkogel, Stoderzinken and Nockberge

Element	Sites		
	Zirbitzkogel	Stoderzinken	Nockberge
pH	3.74 ± 0.03a	4.26 ± 0.11b	4.04 ± 0.06ab
	Total elements (g kg^{-1})		
N	1.53 ± 0.05b	1.64 ± 0.07b	1.02 ± 0.07a
C	36.3 ± 1.0b	41.0 ± 2.0b	24.2 ± 2.0a
C/N	23.7	25.0	23.7
	Total elements (mg g^{-1})		
Al	5.50 ± 0.34a	7.28 ± 1.27a	12.11 ± 0.98b
Ca	2.22 ± 0.17a	11.78 ± 1.10b	4.71 ± 0.58a
Fe	9.58 ± 0.83a	9.31 ± 1.56a	28.12 ± 1.54b
K	0.90 ± 0.03a	0.81 ± 0.07a	1.07 ± 0.16a
Mg	1.03 ± 0.07a	1.21 ± 0.14a	3.83 ± 0.29b
Mn	0.15 ± 0.01a	0.34 ± 0.06ab	0.51 ± 0.04b
Na	0.10 ± 0.01b	0.07 ± 0.01a	0.13 ± 0.02b
P	0.98 ± 0.04a	0.79 ± 0.04a	1.06 ± 0.03a
S	1.52 ± 0.06b	1.51 ± 0.04b	0.94 ± 0.06a
	Exchangeable elements (mg g^{-1})		
Al	0.46 ± 0.04b	0.12 ± 0.02a	0.50 ± 0.04b
Ca	1.44 ± 0.10a	8.35 ± 0.67b	1.60 ± 0.15a
Fe	0.22 ± 0.02b	0.07 ± 0.01a	0.26 ± 0.02b
K	0.35 ± 0.02b	0.25 ± 0.01ab	0.17 ± 0.01a
Mg	0.16 ± 0.01a	0.44 ± 0.02b	0.38 ± 0.04ab
Mn	0.03 ± 0.01a	0.11 ± 0.02b	0.13 ± 0.01b
Na	0.09 ± 0.01b	0.07 ± 0.01b	0.04 ± 0.01a
P	0.05 ± 0.01a	0.05 ± 0.01a	0.05 ± 0.01a

et al. 2015), unlike in young stands (Mandolini et al. 2022), in mature stands *Suillus plorans* is not highly abundant either based on ectomycorrhizal tips and less based on individuals. It does, however, have a high frequency occurring on 80 percent of all plots sampled at Nockberge and Stoderzinken. Similarly, Mandolini et al. (2022) found *Suillus plorans* occurred on all sites investigated at a low abundance. *Tylospora fibrillosa* and *Lactarius rufus* were the most dominant taxa across all sites. Using total soil DNA, Probst et al. (2024) found that highest number of amplicon reads were for *Russula decolorans,* producing up to 40% of all reads at one site. This suggests that based on our data and that of Probst et al. (2024), *Russula decolorans* is a key species in *Pinus cembra* forests. Probst et al. (2024) also reported that an unidentified *Rhizopogon,* but not *Suillus,* were part of the core taxa in soils of *Pinus cembra* forests. In our analysis we identified *Rhizopogon sp. 'subgenus Amylopogon*, and a member of this sub-genus *Rhizopogon aff. Salebrosus,* both of which have been shown to occur in North America (Dowie et al. 2017) rather than the European Alps. To our knowledge this is the first documented occurrence of this taxa in the European Alps. Although specialist fungi such as *Suillus* and *Rhizopogon* occurred in the community, dominate taxa were generalists such as *Tylospora fibrillosa*, *Lactarius rufus*, and various *Russula* spp. This suggests that despite the distinct ecology of *Pinus cembra* the composition ectomycorrhizal community is less exceptional. However, although *Suillus* and *Rhizopogon* occurred at low abundance they had a comparatively high frequency, which is a striking feature of the communities. Rosinger et al. (2018) showed that occurrence was often a better measure of ecological importance than abundance at single sites. However, based on enzyme activity, abundance within a population was shown to be the most significant factor of ecological importance in EM communities (Wang et al. 2017; Otgonsuren et al. 2020).

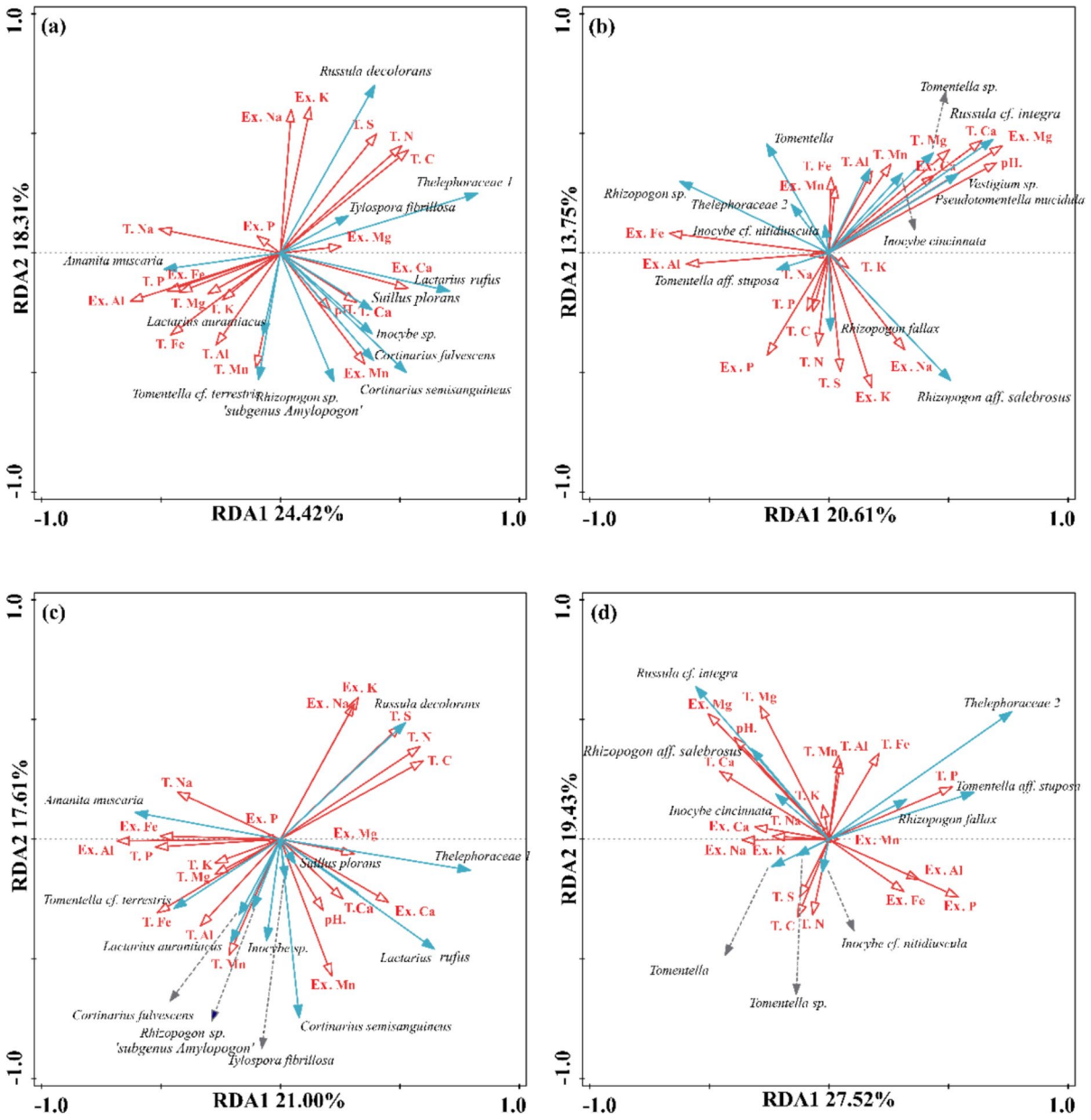


Fig. 7 Redundancy analysis (RDA) of the relationships in soil variables and abundance of EM fungal taxa vital (**a**, **b**) and sub-vital tips (**c**, **d**) in the study areas. For clarity the fungal taxa are presented as two groups. Ex. represents exchangeable and T. total element concentrations

Status of ectomycorrhizal fungal root tips across vital and sub-vital tips

Across all sites, most taxa showed a predominance of vital root tips. For instance, *Suillus plorans* consistently exhibited a very low proportion (< 1%) of sub-vital tips. Similarly, *Rhizopogon sp.*, *Pseudotomentella mucidula*, and *Vestigium sp.* also had a low proportion of sub-vital root tips. The absence of sub-vital tips suggest that these taxa have longer lifespans, compared to the taxa that have a higher proportion of sub-vital tips such as *Amanita muscaria* and *Inocybe* (Fernandez et al. 2013; Kou et al. 2017). Other studies have shown that the

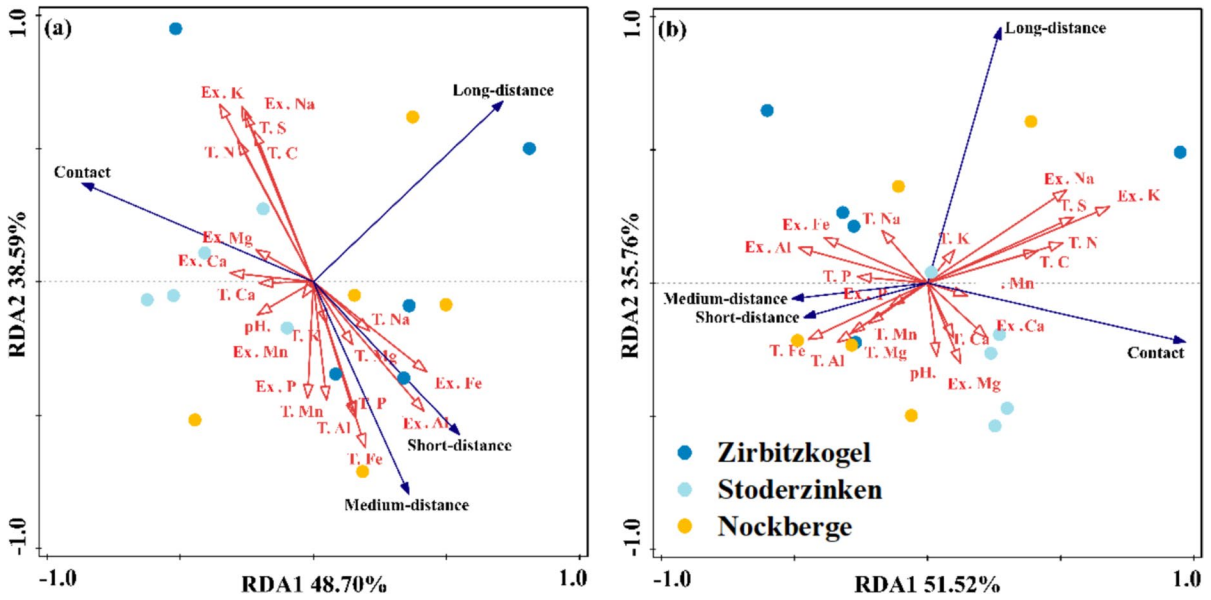


Fig. 8 Redundancy analysis (RDA) abundance of EM fungal exploration types with soil properties across Zirbitzkogel, Stoderzinken and Nockberge. **a**, Taxa abundance based taxa individuals; **b**, Taxa abundance based vital tips. Ex. represents exchangeable and T. total element concentrations

lifespan may differ between ectomycorrhizal taxa (Lukac and Godbold 2011; Fernandez et al. 2013). However, a high proportion of sub-vital ectomycorrhizal tips may also reflect a slower rate of decomposition and disappearance. Interestingly, Stoderzinken showed the highest proportion of vital tips, and Nockberge the lowest value. Given the otherwise similar community composition, this suggests that soil factors, particularly Ca availability and pH, may enhance root tip longevity.

Influence of soil properties on ectomycorrhizal fungal taxa

Soil properties significantly contribute to the composition of EM fungal communities at high altitudes and are a primary factor influencing EM community structure across different ecosystems (Adamo et al. 2021; Koizumi et al. 2018). Numerous previous reports have emphasized that particularly pH, and total C, N, and P, are the primary driving forces shaping EM fungal communities (Koizumi et al. 2018; Tedersoo et al. 2020). In the present study, abundance of EM taxa correlated with soil mineral nutrient composition, such as exchangeable Ca and Al. Stoderzinken showed a higher soil pH compared to the other two sites, reflecting the calcareous bedrock. These soil chemical differences indicate a clear contrast between the calcareous Stoderzinken site and the two silicate sites, which are mirrored in the composition and functional traits of the EM fungal community. A positive correlation between relative abundance of *Suillus plorans*, *Lactarius badiosanguineus*, and *Lactarius rufus* with pH, total Ca and exchangeable Ca was shown. The high Ca concentrations appear to play a role in shaping the EM fungal community, as supported by the studies of Timling et al. (2012) and Scattolin et al. (2008) in artic or alpine ecosystems, respectively. In addition, the EM community at Stoderzinken also contained a higher proportion of contact exploration types associated to pH and exchangeable Ca. *Amanita muscaria* showed a positive correlation with exchangeable Fe and Al and was not present at Stoderzinken. While total concentrations did not always translate into higher exchangeable nutrient availability, these patterns underline the role of soil geochemistry in structuring EM communities. Importantly, even within genera such as *Cortinarius*, *Lactarius* or *Russula*, different taxa may show contrasting soil preferences, other studies have reported that species from these genera tend to be more abundant in acidic soils (Kohler et al., 2015). Only using a high sampling

effort is it possible to detect such differences in EM community structure, however in our study the high sampling effort comes at the expense of the number of sites investigated. Clearly, our conclusions are drawn on only one calcareous site and two silicate sites, but the differences between sites are highly robust. Additionally, we cannot rule out that other cryptic factors associated with bedrock type may have influenced the EM community structure.

Conclusion

This study showed differences in patterns in EM fungal communities across three alpine *Pinus cembra* forest sites—Zirbitzkogel, Stoderzinken, and Nockberge. While overall fungal composition remained similar between sites, notable differences in taxa abundance and frequency were observed. Nockberge exhibited the highest species richness and diversity, whereas Stoderzinken was characterized by lower diversity. Soil properties were associated with EM fungal composition and exploration types, where exchangeable Ca and Mg were found to be the most influential factors shaping EM fungal communities. Calcium-rich soils favored contact exploration types in Stoderzinken. *Tylospora fibrillosa* and *Lactarius rufus* emerged as dominant taxa across all sites, underlining their ecological importance in these alpine environments.

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Data availability All data produced or examined in this study are provided in this published article.

Declarations

Conflict of interests Authors declare no conflicts of interests.

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