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High N_2 fixation rates in mycorrhizal tubercles on pine roots

Wenyi Xu [a,*], Shahid Mahmood [b], Björn Lindahl [a], Marie Spohn [a]

[a] Department of Soil and Environment, Swedish University of Agricultural Sciences (SLU), Lennart Hjelms väg 9, 75007 Uppsala, Sweden
[b] Department of Forest Mycology and Plant Pathology, Uppsala BioCenter, Swedish University of Agricultural Sciences (SLU), Box 7026, 75007 Uppsala, Sweden

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ABSTRACT

Tuberculate ectomycorrhizae (tubercles) are a type of mycorrhizal association, in which the fungus and root combine to form nodular structures. Diazotrophs associated with these structures could fix atmospheric N_2 and contribute to N supply for plants in boreal forest ecosystems. Yet, very little is known about N_2 fixation associated with tubercles.

We quantified N_2 fixation rates associated with tubercles of Scots pine (*Pinus sylvestris*) and in the surrounding soils, and identified diazotrophs associated with the tubercles. For this purpose, pine seedlings were grown in microcosms for three years and N_2 fixation was measured using ^{15}N-N_2. We found that tubercles of *Pinus sylvestris* were formed by *Suillus bovinus*. The N_2 fixation rate associated with the tubercles from one microcosm was particularly high (7587 ng N $g^{-1}h^{-1}$) and was 35-fold higher than the mean rate of the other microcosms. The tubercles exhibited significantly higher N_2 fixation rates than the soils. A total of 26 orders of diazotrophs, belonging to nine phyla (*Actinomycetota*, *Bacteroidota*, *Bacillota*, *Cyanobacteria*, *Desulfobacterota*, *Euryarchaeota*, *Nitrospirota*, *Pseudomonadota* and *Thermodesulfobacteriota*) was associated with the tubercles. In the tubercles with exceptionally high N_2 fixation, *Euryarchaeota* and specifically *Methanosarcinales* dominated the diazotrophic community. In conclusion, the tubercles of Scots pine harbored active diazotrophs and exhibited high N_2 fixation rates, potentially serving as a significant N source in boreal forests.

1. Introduction

Nitrogen (N) is usually a limiting nutrient for plant productivity in boreal forests. Due to low atmospheric N deposition, biological N_2 fixation serves as a major N source (Bradshaw and Warkentin, 2015; Jarvis and Linder, 2000). Non-symbiotic N_2 fixation, such as moss-associated N_2 fixation and free-living N_2 fixation in soils, can be a larger N input in boreal forests than symbiotic N_2 fixation, because symbiotic N_2 fixation plants are rare in boreal forests (DeLuca et al., 2008; Dynarski and Houlton, 2018; Gundale et al., 2012; Vázquez et al., 2025; Vázquez and Spohn, 2025). However, these N sources (atmospheric N deposition, and N_2 fixation associated with mosses and in soils) cannot always meet the N needs for plant growth (Ladanai et al., 2007), suggesting that additional N input could contribute to the overall N supply. In boreal forests, tree roots are associated with ectomycorrhizal fungi. Some of them form structures called tuberculate ectomycorrhizae (tubercles), and these fungal structures have been reported to harbor diazotrophs (Frey-Klett et al., 2007; Li et al., 1992; Paul et al., 2007). Thus, diazotrophs associated with tubercles could fix atmospheric N_2 and play an important role in N supply for plants in these ecosystems. Yet, very little is known about N_2 fixation associated with tubercles.

Tubercles have primarily been documented in associations between host species in the genera *Pinus* and *Pseudotsuga* and fungal partners in the genera *Suillus* and *Rhizopogon* (from the order *Boletales*) (Li et al., 1992; Paul et al., 2007). *Suillus* species are well-known as effective symbionts that facilitate host seedling establishment and development (Jenkins et al., 2018) and can act as hosts to a diverse range of bacteria that colonize both living hyphae and mycorrhizal structures (Izumi et al., 2006; Lofgren et al., 2024). A similar association has also been observed between *Quercus* and *Boletus rubropunctus* (now *Hemileccinum rubropunctum*), another member of the order *Boletales* (Smith and Pfister, 2009). These findings suggest that tubercles are specific to a relatively small group of fungal species within *Boletales*.

The tuberculate structures are composed of highly branched short roots tightly packed within a common fungal mantle, with some morphological similarities to root nodules (Li et al., 1992; Paul et al., 2007). These structures may provide a favorable microenvironment for diazotrophs as they provide protection against environmental stress and

* Corresponding author.
E-mail address: wenyi.xu@slu.se (W. Xu).

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competitors (Yang et al., 2016) as well as carbohydrates from the host plants (Mohd-Radzman and Drapek, 2023; Wurzburger, 2016). For example, Lodgepole pine (*Pinus contorta*) has been reported to feature tuberculate structures where diazotrophs reside and exhibit high rates of N_2 fixation (Chapman and Paul, 2012; Paul et al., 2013). The N_2 fixation occurring within these structures can significantly contribute to the N requirement of Lodgepole pine (Paul et al., 2013). Due to unique structural and functional characteristics of the tubercles, the diazotrophs associated with them may fix N_2 at a higher rate than diazotrophs in the surrounding soil that are usually constrained by carbon (C) availability and environment stress (Dittmann et al., 2025; Smercina et al., 2019).

Scots pine (*Pinus sylvestris*) belongs to the same genus as *Pinus contorta*, which features tubercles previously known for high rates of N_2 fixation (Paul et al., 2007). It is the most widely distributed *Pinus* species in the world and is a dominant tree species in the N-limited Scandinavian boreal forests (Wachowiak et al., 2022). However, it remains unknown whether diazotrophs associated with the tuberculate structures of Scots pine and what the rate of N_2 fixation is.

The aims of the study were (1) to quantify N_2 fixation rates associated with the tuberculate structures formed by Scots pine roots in association with species in the *Boletales* in comparison to N_2 fixation rates in the surrounding soil, (2) to identify diazotrophs associated with these structures, and (3) to determine the fungi that form the tuberculate structures. We tested the hypothesis that active diazotrophs associated with the tuberculate structures of Scots pine fix N_2 at a higher rate than diazotrophs in the soil. We used pine seedlings grown in microcosms, since this allowed us to isolate the trees and tubercles from the soil and incubate the tubercles attached to the living seedings, which supplies them with C, during the determination of the N_2 fixation rate. Isolating trees and tubercles from the soil is practically impossible in forest ecosystems where the mycorrhizal fungi and roots of old and young trees form a dense network.

2. Materials and methods

2.1. Plant and soil preparation

Certified *Pinus sylvestris* L. seedlings were obtained from Svenska Skogsplantor (https://www.skogsplantor.se) a nursery unit of the forest company Sveaskog (https://www.sveaskog.se). The seedlings were three months old, growing in a peat-based growth substrate, and this particular batch had not been treated with any biocides. The seedling trays were placed in a phytotron under a 250 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) regime with an 18 h: 6 h light–dark cycle and a day-night temperature of 18 °C: 16 °C. The seedlings were maintained under these conditions for approximately six weeks before use and were irrigated weekly with deionized water.

Soils were collected from the organic (O), eluvial (E), and illuvial (B) horizons of a podzol located at Ivantjärnsheden, Jädraås in central Sweden (60°49′N, 16°30′E; altitude 185 m). This site lies at the transition between the Swedish boreal and boreo-nemoral zones (Persson, 1980; Mielke et al., 2022). The forest is a homogenous, evenly aged (160 years) stand of *Pinus sylvestris* L., with occasional *Picea abies* (L.) Karst. individuals. The understory vegetation is dominated by the ericaceous dwarf shrubs *Vaccinium vitis-idaea* L. and *Calluna vulgaris* (L.) Hull, with a sparser cover of *Empetrum nigrum* L., *Vaccinium myrtillus* L., and feather moss *Pleurozium schreberi* (Brid.) Mitt. (Bråkenhielm and Persson, 1980).

The soil profile consists of a glacifluvial sandy podzol with a surface mor layer (pH 3.0), a pale eluvial horizon, and a rust-red illuvial horizon in the mineral soil (pH 4.4–4.8; (Bringmark, 1980)). The underlying bedrock comprises granites, sedimentary, and volcanic rocks, which are typical of the Fennoscandian region. Organic and mineral horizon soils were homogenized using 5 mm and 3 mm mesh sieves, respectively. Freshly fallen litter, stones, roots, and lichens were removed prior to sieving (Mahmood et al., 2024).

2.2. Microcosms with reconstructed podzol horizons

Microcosms with reconstructed podzol horizons were set up as described by Mahmood et al. (2024). Freshly sieved soils from the different horizons were transferred into six transparent acrylic containers. Each container had holes drilled in the base to allow for aeration and drainage. Three microcosms were prepared with stratified O, E and B horizons (Fig. 1a), which reflect a typical natural boreal forest soil (undisturbed). The other three microcosms had mixed O, E and B horizons (Figs. 1b and 1c), which represent a condition that is caused by whole tree harvesting with stump removal (disturbed soil structure). Before the addition of the soil, two nylon mesh sheets (0.5 mm thick, 0.6 mm pore size) were placed at the base of each microcosm to prevent soil loss through drainage holes and to aid harvesting. In the stratified microcosms, a nylon mesh sheet (1.0 mm thick, 2.0 mm pore size) was placed on the surface of the B horizon soil to maintain horizon separation and aid harvesting (of roots and soil). Then, 800 g of E horizon soil was added. A second nylon mesh of the same type was placed between the E and O horizon soil. In the mixed microcosms, nylon mesh sheets were placed at soil depths corresponding to the interfaces between O, E and B horizons, mirroring the stratified setup. Three pine seedlings were planted at a standard soil depth (3 cm) in each microcosm. Prior to planting, root system of each seedling was washed several times with deionized water to remove residues of peat-based growth substrate. Each microcosm was wrapped in aluminum foil and the soil surface was covered with a thick black plastic sheet. This prevented exposure of developing mycelia to light and also inhibited the growth of algae and mosses. The microcosms were incubated in a growth chamber for three years (Fig. 1d), with a 250 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) 18 h: 6 h, light: dark cycle and day: night temperature of 18 °C: 16 °C, and air humidity of 60 %. Soil moisture was kept constant by gravimetric watering with deionised water. Soil moisture was kept constant by gravimetric watering with deionised water. The positions of the microcosms were randomised weekly to minimize the effects of spatial variability within the chamber.

2.3. Plant and soil sampling

The three pine seedlings were excavated carefully from each microcosm, minimizing damage to roots and tubercles as much as possible. The roots and tubercles of the seedlings were then rinsed to remove adhering soil and gently dried with a paper towel. Some tubercles were collected randomly for the measurement of ^{15}N natural abundance.

From each of the three stratified microcosms, three samples were collected from the 3 cm thick O horizon with a 2-cm-diameter auger, and were subsequently mixed gently into one composite sample. Similarly, from each of the three mixed microcosms, three samples were taken from the same soil depth (3 cm) corresponding to the O horizon in the stratified microcosms, and were subsequently mixed gently into one composite sample.

2.4. Measurement of N_2 fixation

The three seedlings from each microcosm were incubated in an 800 mL jar with ^{15}N enriched artificial atmosphere. We incubated the whole seedlings to ensure that the tubercles attached to the living seedlings can receive photosynthates from the host during the incubation. This allows to measure N_2 fixation rates under realistic conditions and to affect the fungi and diazotrophs as little as possible. Each jar was closed with a gas-tight screw lid, flushed with argon (Ar), then carefully evacuated, and finally filled with 585 mL $^{15}N_2$ (99.8 atom%; Sigma Aldrich co., St. Louis, MO, USA) and 157 mL oxygen (O_2) (Fig. 1e). The Certificate of Analysis of the $^{15}N_2$ gas lot that we used (MBBD6749 produced by Sigma Aldrich in 2023) shows that the N_2 gas is 99.9 % pure and contains < 15 ppm N_2O (see the Certificate of Analysis of the gas lot in the

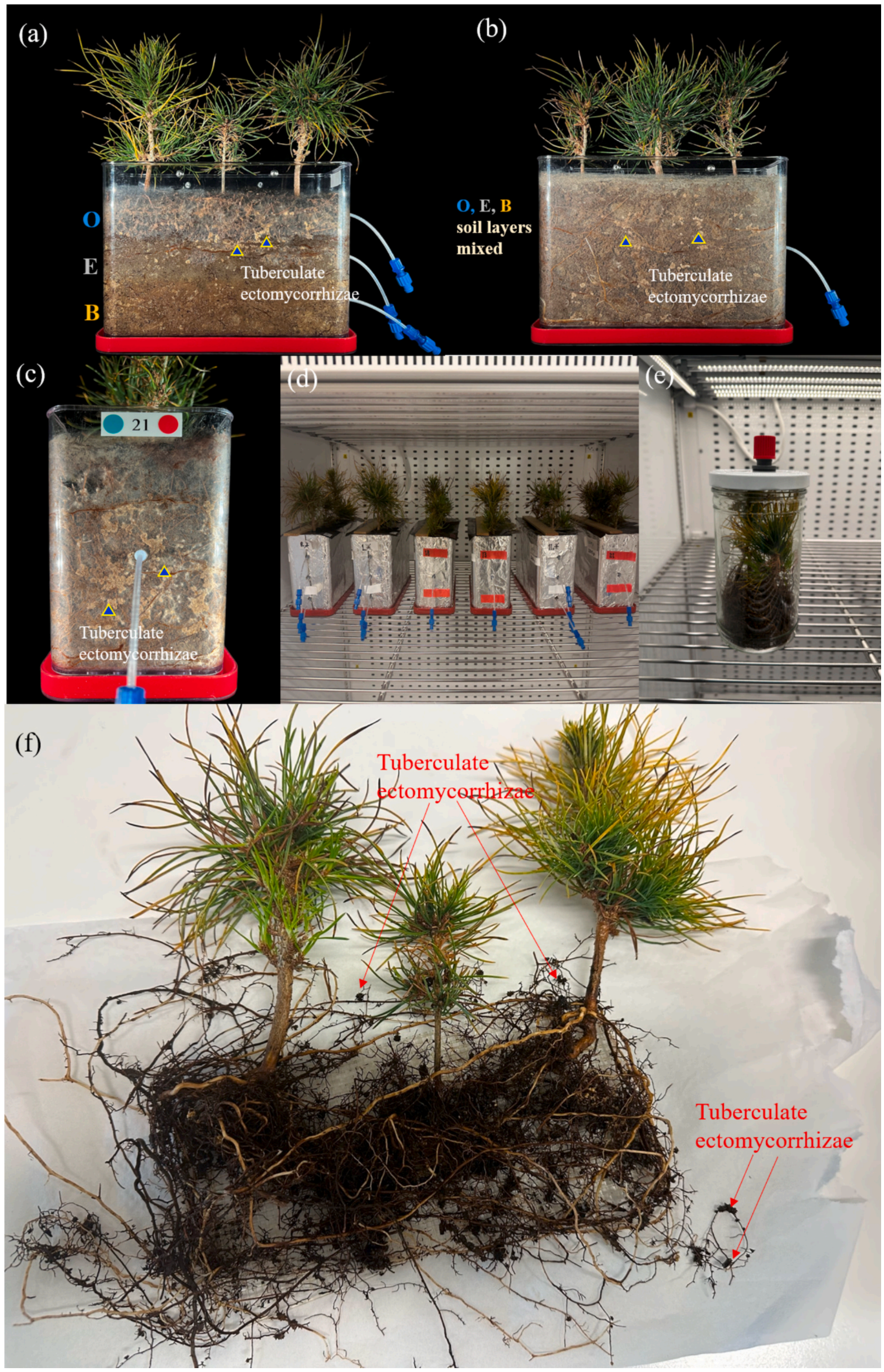

Fig. 1. Scots pine (*Pinus sylvestris*) seedlings were grown in acrylic containers with a reconstructed boreal podzol profile using organic (O), eluvial (E), and illuvial (B) horizon soils (microcosms 1–3) (a) or mixed horizons (microcosms 4–6) (b, c), the six microcosms were incubated in a growth chamber with light (d), the seedlings were incubated in a jar with added $^{15}N_2$ (e), and tubercles (tuberculate ectomycorrhizae) of Scots pine (*Pinus sylvestris*) seedlings (f).

Supplementary Material). All jars were incubated in a climate chamber for 48 h with a 250 μmol m^{-2} s^{-1} PAR 18 h: 6 h, light: dark cycle, air humidity of 60 %, and day: night temperature of 18 °C: 16 °C.

Moist soil (equivalent of 1.5 g dry-mass) from each microcosm was incubated in a 30 mL serum flask with a ^{15}N enriched artificial atmosphere. Each flask was closed with a gas-tight aluminum crimp seal with PTFE/butyl septa, flushed with Ar, carefully evacuated, and finally filled with 25 mL $^{15}N_2$ (99.8 atom%; Sigma Aldrich co., St. Louis, MO, USA) and 3.6 mL O_2. All the flasks were incubated in the dark at 15 °C (simulating soil temperatures over the growing seasons) for 48 h.

At the end of the incubation, the jars or flasks were opened under the fume hood for 5 min. Tubercles of the three seedlings in each jar were collected and pooled per microcosm (Fig. 1f). Some tubercles were not labeled with $^{15}N_2$ but used for natural abundance measurements. The tubercles and soil samples exposed to $^{15}N_2$ (^{15}N labeled) as well as non-exposed samples (natural abundance; unlabeled) were freeze-dried and milled. Subsequently they were analyzed for ^{15}N using a continuous-flow isotope ratio mass Spectrometry of Flash EA 2000 via ConFlo IV open split interface to a Delta V isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).

2.5. DNA extraction and sequencing

DNA was extracted from 0.14 g of frozen tubercle material using the NucleoSpin kit (Macherey-Nagel GmbH & Co, Düren, Germany) according to the manufacturer's protocol (using SL1 lysis buffer without the Enhancer solution). Tissue was homogenized with lysing matrix E (MP Biomedicals) in a Precellys bead beater (Bertin technologies). The DNA concentration was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, MA, USA).

DNA markers containing the ITS2 regions as well as parts of the 18S ribosomal gene, altogether around 900 bp, were amplified in duplicate 50 μL reactions containing 12.5 ng extracted DNA, 0.5 μM of gITS7 (Ihrmark et al., 2012), 0.3 μM of TW13, both elongated with 8 bp sample-unique identification tags, and Phusion Hot Start II High-Fidelity PCR Master Mix according to the manufacturer's instructions (Thermo Fisher Scientific), followed by 25 cycles of 98 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s, and a final extension step of 7 min at 72 °C. Duplicate amplicons were then pooled and purified using AMPure magnetic beads (Beckman Coulter). Adaptor ligation and sequencing on the Pacific Biosciences Revio platform were performed by SciLifeLab NGI (Uppsala, Sweden).

For *nifH*, the primers 19F (Ueda et al., 1995) and R6 (Marusina et al., 2001) were used. The *nifH* was sequenced using a two-step PCR protocol. The first step was performed in duplicate 15 μL reactions containing 3.6 ng extracted DNA, 0.8 μM of the abovementioned *nifH* gene-specific primers supplemented with Nextera adaptor sequences (Illumina Inc, San Diego, CA, USA), 0.2 mM dNTP and 0.025 U/μL DreamTaq DNA polymerase with Green reaction buffer (Thermo Fisher Scientific), supplemented with 0.75 mM $MgCl_2$, followed by 28 cycles of 98 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s, and a final extension step of 10 min at 72 °C. Duplicate amplicons were then pooled and purified using SeraMag magnetic beads (GE Healthcare, Chicago, IL, USA). The second amplification step was performed during eight cycles in duplicate under the same conditions as above using 10 % of the purified product from the first step and 0.2 μM primers with Nextera adapter- and barcoding regions for dual labelling of the fragments. Duplicates were then pooled and purified as above. Purified samples were quantified with a Qubit fluorometer, pooled equimolarly, and sequenced by SciLifeLab NGI (Stockholm, Sweden) on an Illumina MiSeq instrument using the 2x250 bp chemistry.

Fungal ITS2-18S sequences were processed using the SCATA pipeline (https://scata.mykopat.slu.se). After quality filtering, sequences were clustered using a single linkage algorithm with a 98.75 % similarity required to enter clusters. Cluster representatives were identified by comparison to the UNITE database (Abarenkov et al, 2024) using BLAST. The *nifH* sequences were processed using the DADA2 package generating amplicon sequence variants (ASVs) (Callahan et al, 2016). The nucleotide sequences of ASVs were aligned against the updated *nifH* reference sequences based on the database from the Zehr Lab (v. June 2017) using BLAST (Heller et al., 2014; Moynihan and Reeder, 2023). The alignment threshold is based on the standard parameters of the BLAST method, including % identity, alignment, length, mismatches, number of mismatches and gap openings, query and subject start and end positions, E-value, and bit score. The alignment threshold is set to 80 % percent identity and an E-value of 1×10^{-6}. Both *nifH* and *chlL/bchL* sequences are included in the reference database (Moynihan and Reeder, 2023). Therefore, the query sequences that match the *chlL/bchL* sequences can be identified and excluded. Raw sequence read files are available in the short Read Archive of NCBI (bioproject ID, PRJNA1337312). The average and minimum number of *nifH* sequence reads was 60,574 and 16,421 respectively. The relative read abundance of the certain taxa was referred to relative abundance of the certain taxa.

2.6. Calculations and statistics

The N_2 fixation rate (in ng N g dry tubercle mass^{-1}h^{-1} or ng N g dry soil mass^{-1}h^{-1}) was calculated using an isotope mixing model (Zechmeister-Boltenstern, 1996):

$$N_2\,\text{fixation rate}\left(\text{ng N g}^{-1}\,\text{h}^{-1}\right) = \text{TN}\left(\text{mg N g}^{-1}\right) \times \frac{\left(\text{atom\%}^{15}\text{N}_{\text{labeled}} - \text{atom\%}^{15}\text{N}_{\text{unlabeled}}\right)}{100 \times t\,(\text{h})} \times 10^6$$

where TN is the total N of the tubercle or soil, $^{15}N_{labeled}$ is the percentage of ^{15}N atoms in the labeled samples, $^{15}N_{unlabeled}$ is the percentage of ^{15}N atoms in the unlabeled samples, and t is the incubation time (i.e., 48 h).

Prior to statistical analysis, we inspected the QQ-plots (quantile–quantile plots) and used Shapiro-Wilk normality test to check data for normal distribution. Significant differences in N_2 fixation rates associated with tubercles and in the soil between stratified and mixed microcosms were tested using a two-tailed *t*-test. A one-tailed paired *t*-test was used to assess whether N_2 fixation rates associated with tubercles were significantly higher than those in soils, because each tubercle-soil pair was from the same microcosm.

3. Results

The rate of N_2 fixation in the topsoil of the stratified microcosms (1 – 3; only organic horizon) was significantly higher than that of the mixed microcosms (4 – 6; mixed organic and mineral horizons; $p < 0.01$; Table S1). The mean N_2 fixation rate associated with the tubercles (excluding one outlier; 1727 ng N g^{-1}h^{-1}) was 19-fold higher than the mean rate in the soil (92 ng N g^{-1}h^{-1}) from all the microcosms ($p = 0.03$) and was 16-fold higher if the outlier was not removed ($p = 0.07$; Fig. 2). The rate of N_2 fixation associated with tubercles was higher by a factor of 35 in one of the microcosms (number 1; 7587 ng N g^{-1}h^{-1}) compared to the mean of the five others (217 ng N g^{-1}h^{-1}; Fig. 3).

The average total C and N contents were 320.5 and 14.6 g kg^{-1} in tubercles from all the microcosms, 347.5 and 8.5 g kg^{-1} in the top soil of the stratified microcosms, and 27.2 and 0.8 g kg^{-1} in the top soil of the mixed microcosms, respectively (Table S2).

In tubercles from microcosms 1 – 4, *Suillus bovinus* (Pers.) Roussel (1898) dominated the fungal community (59 % – 97 %), followed by unidentified *Archaeorhizomyces* species (2 % – 32 %; Fig. 4). In microcosms 5 and 6, the fungal community was dominated by *Archaeorhizomyces* sp. (58 % and 68 %, respectively), while the remaining fungi were present at low relative abundances (Fig. 4).

NifH genes from 26 prokaryotic orders, belonging to nine phyla

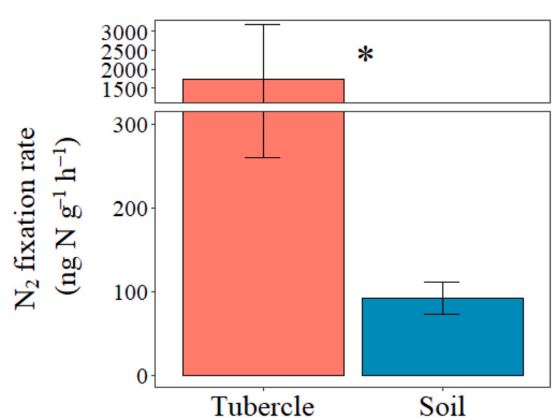

Fig. 2. N_2 fixation rates associated with tubercles (tuberculate ectomycorrhizae) and in the soils from all the microcosms. Bars show mean (± standard error) of replicates (n = 5 and 6 for the tubercle and soil samples, respectively). One outlier of tubercle samples is excluded. A significant difference in the rates between tubercles and soils is shown as * and tested by a one-tailed pairwise *t*-test.

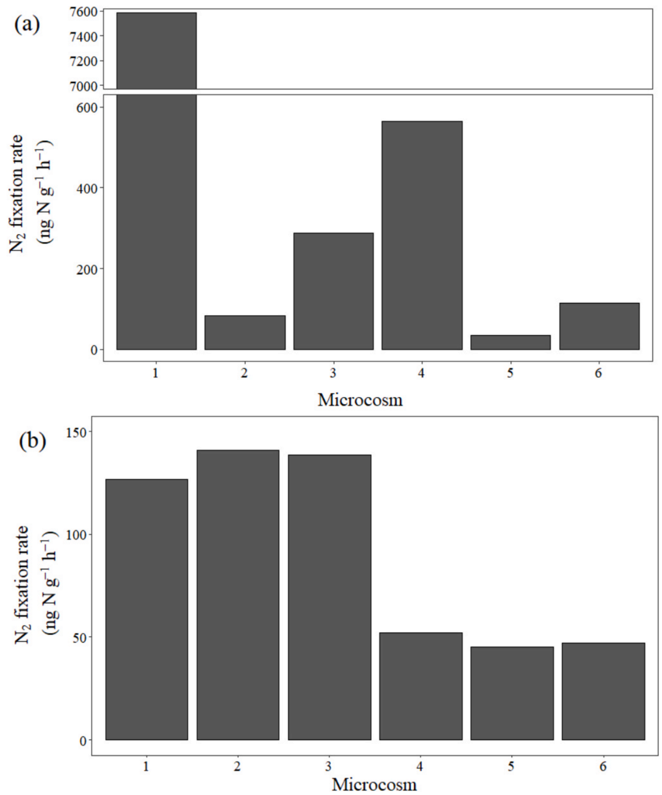

Fig. 3. N_2 fixation rates associated with tubercles (tuberculate ectomycorrhizae) (a) and N_2 fixation rates in the top 3 cm of the soils (b) from each of the six microcosms. Stratified horizons (microcosms 1–3) and mixed horizons (microcosms 4–6).

(*Actinomycetota, Bacteroidota, Bacillota, Cyanobacteria, Desulfobacterota, Euryarchaeota, Nitrospirota, Pseudomonadota,* and *Thermodesulfobacteriota*), were identified in the tubercles from the microcosms (Fig. 5 and Table S3). In microcosm 1 (with particularly high N_2 fixation), *Euryarchaeota* dominated the diazotrophic community at the phylum level (75 %), followed by *Pseudomonadota* (23 %; Fig. 5a). Within the phylum *Euryarchaeota*, only the order *Methanosarcinales* was detected (Fig. 5b). In the other microcosms (5 – 6), the diazotrophic community at the phylum level was dominated by *Pseudomonadota* (86 % – 99 %; Fig. 5a). The phylum *Pseudomonadota* consisted of 14 detected orders, with *Hyphomicrobiales* being the most dominant (Fig. 5b). The diazotrophic community of the tubercles from the stratified microcosms differed more strongly among the microcosms than the one of the tubercles from the mixed microcosms (Fig. 6).

4. Discussion

We found that the tubercles fixed N_2 at a significantly higher rate than the soils, supporting our hypothesis (Fig. 2). The N_2 fixation rates associated with tubercles of most microcosms were comparable with the commonly reported rates by moss-associated diazotrophs (190 ng N $g^{-1}h^{-1}$) in boreal forests (DeLuca et al., 2002; Rousk et al., 2013a). However, the values were twice as high as the values (98.5 ng N $g^{-1}h^{-1}$; excluding the extremely high values) reported for tubercles formed by *Pinus contorta* and *Suillus tomentosus* (Paul et al., 2007) and much higher than the values (15 ng N $g^{-1}h^{-1}$) reported for tubercles formed by *Pseudotsuga menziesii* and *Rhizopogon vinicolor* (Li et al., 1992). This discrepancy may be attributed to the high spatial and temporal variability in N_2 fixation of the tubercles. In addition, high soil mineral N availability is known to suppress nitrogenase activity (Bellenger et al., 2020; Wani et al., 1997), and tubercles occurring in N-rich stands may exhibit lower N_2 fixation rates (Li et al., 1992).

The high rates associated with tubercles were likely due to the unique structural and functional characteristics of the tubercles. For example, their large surface area relative to other mycorrhizal types may promote the colonization and growth of diazotrophs, while the structure itself also serves as a nutrient reservoir (Borken et al., 2016; Smith and Pfister, 2009). The process of N_2 fixation is oxygen-sensitive and energy-intensive (Bellenger et al., 2020), and thus the low oxygen conditions within the tuberculate structures, along with the C substrates provided by the host plants, can create a favorable environment for diazotrophic activity (Mohd-Radzman and Drapek, 2023; Wurzburger, 2016). In addition, it might be that low-oxygen conditions are created by the ectomycorrhizal fungi, which consume oxygen, and by the physical structure of the tubercles that limit oxygen diffusion, similar to the protective role of nodules of legumes (Minchin, 1997).

To maintain active tubercles with a continuous supply of photosynthates from the host plants, the whole seedlings were incubated in jars with added $^{15}N_2$. However, we cannot rule out the possibility that N_2 fixation also occurred in other plant compartments (Shalovylo et al., 2024). For example, active diazotrophs have been detected in Scots pine needles and roots (Bizjak et al., 2023; Nilsson et al., 2024; Padda et al., 2019; Puri et al., 2018; Różycki et al., 1999), raising the possibility that fixed ^{15}N from the foliage and roots could be translocated to the tubercles. Nevertheless, given the low N_2 fixation rates previously reported for needles (0.84 ng N $g^{-1}h^{-1}$) and roots (0.4–4 ng N $g^{-1}h^{-1}$)

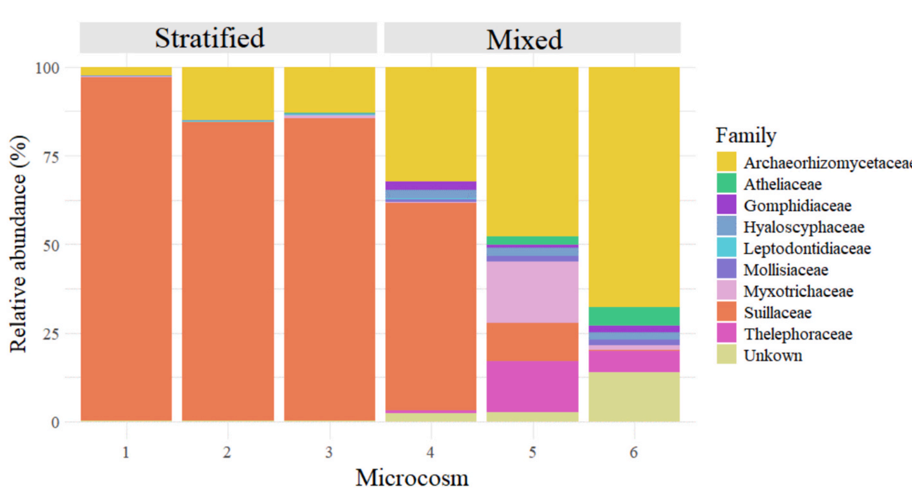

Fig. 4. Relative abundance of fungal community at the family rank associated with tubercles (tuberculate ectomycorrhizae) from each of the six microcosms. Stratified horizons (microcosms 1–3) and mixed horizons (microcosms 4–6).

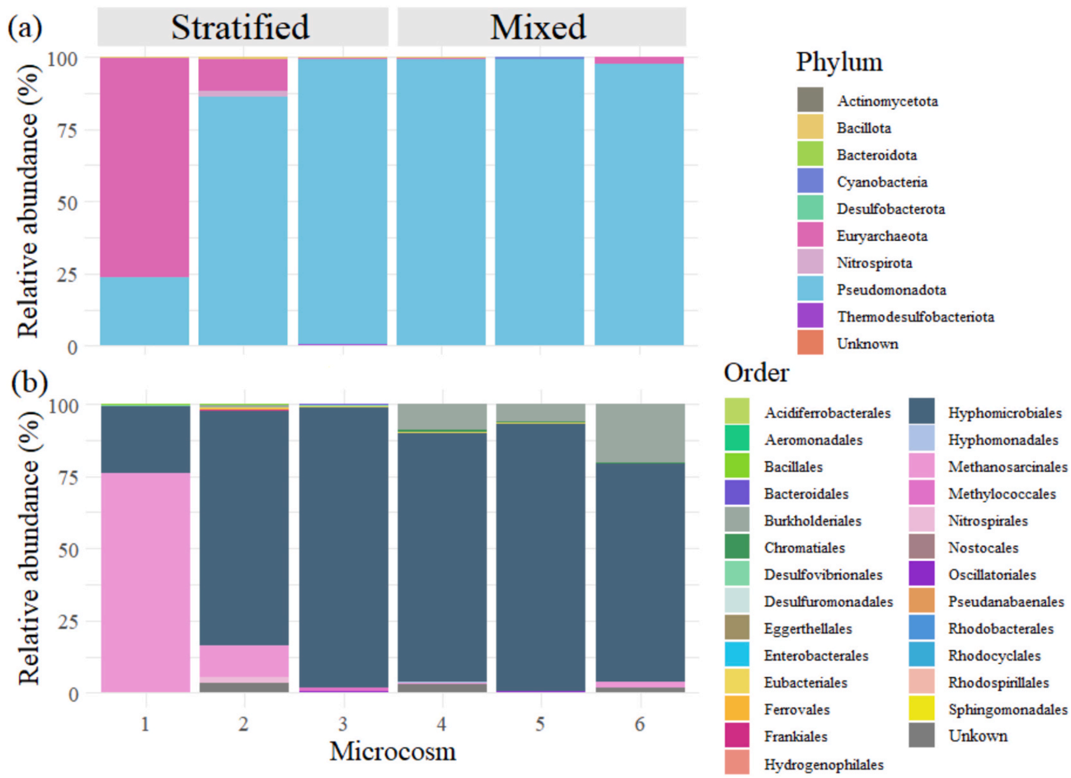

Fig. 5. Relative abundance of diazotrophic community (*nifH*) at the phylum rank (a) and at the order rank (b) associated with tubercles (tuberculate ectomycorrhizae) from each of the six microcosms. Stratified horizons (microcosms 1–3) and mixed horizons (microcosms 4–6).

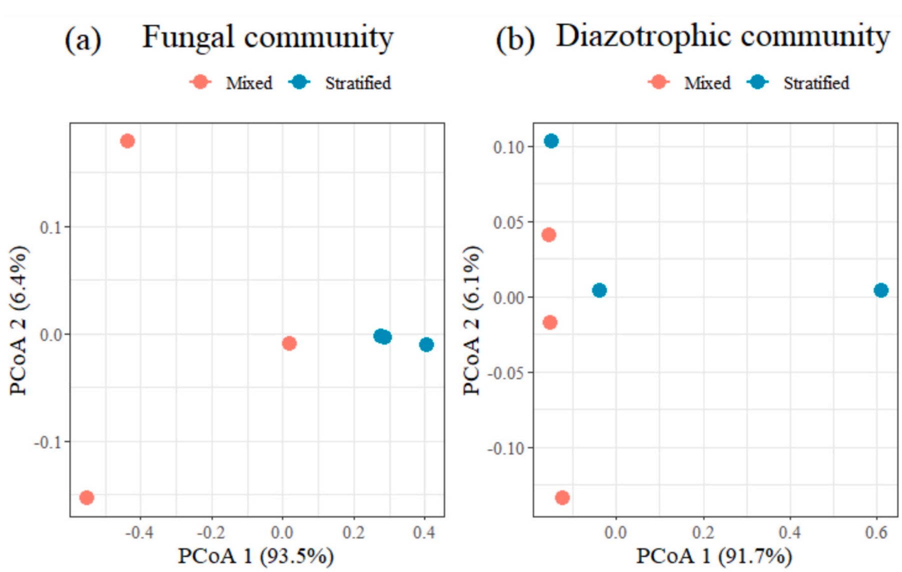

Fig. 6. A principal coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity for fungal community composition at the family rank (a) and diazotrophic community composition at the order rank (b) associated with tubercles (tuberculate ectomycorrhizae) from the microcosms with stratified soil horizons or mixed horizons.

(Bizjak et al., 2023; Nilsson et al., 2024), it is very likely that the majority of the ^{15}N enrichment observed in tubercles originated from N_2 fixation occurring locally within these structures. In addition, potential contamination of commercial $^{15}N_2$ gas with $^{15}NH_3$ cannot be excluded. However, the minimal ^{15}N enrichment observed in the tubercles from microcosm 5 can be considered as the upper boundary for possible contamination, supporting that the higher levels of ^{15}N incorporation in the other microcosms reflect N_2 fixation activity.

In accordance with our hypothesis, we found 26 orders of diazotrophs, belonging to nine phyla, associated with the tubercles. Previously, only the orders *Hyphomicrobiales* and *Paenibacillales*, belonging to the phyla *Pseudomonadota* and *Bacillota*, have been reported in tubercles of *Pinus contorta* (Paul et al., 2013). This detection of a limited number of phyla in previous studies might be due to the use of culture-dependent techniques in these studies (Paul et al., 2013). *Hyphomicrobiales* (a group that includes rhizobia) are commonly found in the root nodules of legumes, which share structural similarities with the tubercles (Hirsch et al., 2001; Paul et al., 2013). The phylum

Pseudomonadota, which dominated the diazotrophic community in tubercles from five microcosms, has also been observed at a similarly high relative abundance in boreal forest soils (Tu et al., 2016; Zhao et al., 2020). A number of archaea belonging to the phylum *Euryarchaeota* have been documented to reside in the mycorrhizosphere of *Pinus sylvestris* (Bomberg and Timonen, 2007) and to have the ability to fix atmospheric N_2 (Bae et al., 2018; Riyaz and Khan, 2024). In addition, *Cyanobacteria* were found associated with tubercles, which are well-known to dominate the diazotrophic community associated with the feather mosses and to contribute significantly to the N-input in boreal forests (Renaudin et al., 2022; Rousk et al., 2013b). The orders *Bacillales, Burkholderiales* and *Hyphomicrobiales* associated with the tubercles have previously been isolated from the roots of Scots pine and demonstrated to fix N_2 (Puri et al., 2018; Różycki et al., 1999).

Exceptionally high N_2 fixation activity (7587 ng N $g^{-1}h^{-1}$) was observed associated with tubercles of one of the microcosms, comparable to the maximum value (8008 ng N $g^{-1}h^{-1}$) reported for tubercles of *Pinus contorta* (Paul et al., 2007). It also exhibited a higher abundance of *Methanosarcinales*, within the phylum *Euryarchaeota*, compared to the other microcosms, which may be related to the elevated N_2 fixation activity. Although *Methanosarcinales* are typically known for their role in methanogenesis, some members of this group have also been shown to harbor nitrogenase genes and possess the ability to fix atmospheric N_2 (Bae et al., 2018; Ehlers et al., 2002; Kouzuma et al., 2015; Prasse et al., 2017; Riyaz and Khan, 2024), and their involvement in N_2 fixation seems plausible. *Methanosarcinales* have been detected in the roots of several tree species, including Norway spruce (*Picea abies)*, Scots pine (*Pinus sylvestris*), silver birch (*Betula pendula*), and black alder (*Alnus glutinosa*) (Bomberg et al., 2011; Bomberg and Timonen, 2009; Putkinen et al., 2021). Furthermore, Bomberg and Timonen (2007) found that mycorrhizal colonization of Scots pine roots increased archaeal abundance and diversity in the rhizosphere, with most of the archaeal sequences belonging to *Euryarchaeota*. These archaea are thought to benefit from low-oxygen microenvironments within the tuberculate structures and from C compounds supplied by the roots (Bomberg et al., 2011). Together, these factors may create favorable conditions that support archaeal N_2 fixation in tubercles.

However, our data do not allow us to definitively attribute the high N_2 fixation rate to *Methanosarcinales* alone. *Hyphomicrobiales* (a group that includes rhizobia) were present in all microcosms and may have been more metabolically active in the microcosm with particularly high N_2 fixation rate, possibly influenced by unmeasured abiotic factors such as localized oxygen gradients, C substrate availability, or other microenvironmental conditions (Bomberg et al., 2011; Riyaz and Khan, 2024). It is also possible that synergistic interactions among microbial taxa can play a role. For instance, the abundance of *Methanosarcinales* might have indirectly stimulated N_2 fixation by *Hyphomicrobiales* through *e.g.* syntrophic cooperation, which has been commonly observed in microbial consortia under nutrient-limited conditions (Kato and Watanabe, 2010; Kouzuma et al., 2015; Thieringer et al., 2023). Notably, N_2 fixation has been reported in methanotrophic archaea of the ANME-2 lineage, which engage in syntrophic cooperation with bacteria (Offre et al., 2013; Riyaz and Khan, 2024). In such systems, N_2 fixation is energetically supported by methanogenesis, as the energy-requiring nitrogenase activity is coupled with the energy-releasing process of methane metabolism (Bae et al., 2018).

The observed N_2 fixation rates associated with tubercles highlight their potential ecological significance as a N source in nutrient-limited boreal forests. The tubercles exhibited much higher N_2 fixation rates than the surrounding soils, suggesting that they may function as hotspots of N_2 fixation in boreal forest ecosystems. The variability in N_2 fixation rates among tubercles further suggests that this contribution is context-dependent, likely influenced by factors such as microenvironmental conditions, host plant physiological status, and microbial community composition (Mohd-Radzman and Drapek, 2023; Smercina et al., 2019). Such spatial and temporal heterogeneity may play a critical role in regulating N inputs at the landscape scale. Thus, how widespread tubercles are and what factors affect the variability should be investigated in the future.

5. Conclusion

Tubercles formed by *Pinus sylvestris* and *Suillus bovinus* harbored active diazotrophs, and exhibited higher N_2 fixation rates than the soils. Notably, the tubercles from one microcosm showed exceptionally high N_2 fixation activity (7587 ng N $g^{-1}h^{-1}$), which may be due to a high abundance of *Methanosarcinales*. It is also possible that *Hyphomicrobiales* that were present in all microcosms, were more metabolically active in this microcosm, potentially triggered by the high abundance of *Methanosacrinales* to fix more N_2 through synergistic interactions. Given the limitations of our current dataset (e.g., absence of gene expression or metabolite profiling), we cannot conclusively determine which taxa were functionally responsible for the observed N_2 fixation. Future research using single-cell analyses could help to reveal the active diazotrophs and uncover potential interspecies interactions driving N_2 fixation in tubercles.

CRediT authorship contribution statement

Wenyi Xu: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Shahid Mahmood:** Writing – review & editing, Methodology. **Björn Lindahl:** Writing – review & editing, Formal analysis. **Marie Spohn:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2025.117643.

Data availability

Data will be made available on request.

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